IL-17 AND TH17 RESPONSES TO HUMAN *HELICOBACTER PYLORI* INFECTION AND THEIR ASSOCIATION WITH DISEASE

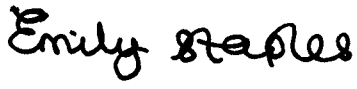
Dr Emily Staples

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, February 2013

MEDICAL LIBRARY
QUEENS MEDICAL CENTRE

DECLARATION

Unless otherwise acknowledged, the work presented in this thesis is my own; no part has been submitted for another degree at the University of Nottingham or any other institute of learning.



Emily Staples

21 February 2013

ABSTRACT

Helicobacter pylori (*Hp*) is a major cause of peptic ulcer disease (PUD) and gastric cancer, yet the infection remains asymptomatic in most people. One factor that influences the outcome of *Hp* infection is the host immune response. Expression of the immune-stimulating cytokine interleukin-17 (IL-17) is increased in the human *Hp*-infected gastric mucosa, but its cellular source and role in pathology are unclear.

In this study dendritic cell cytokine responses to *Hp* stimulation were studied, and relative IL-12p70 and IL-23 concentrations compared, to assess the potential of *Hp* to promote differentiation of IL-17-secreting T-helper cells (Th17). The effect of *Hp* virulence factors on cytokine secretion was assessed and monocyte-derived DC (MoDCs) and $CD1c^+$ myeloid DC (MyDC) responses compared. MoDCs produced high concentrations of IL-12p70 upon *Hp* stimulation. There was also an IL-23 MoDCs response, but this was >10-fold lower than the IL-12p70 response. Both IL-12p70 and IL-23 responses were significantly reduced when *Hp* isogenic mutants for the virulence factor dupA were used, although the effect on MoDC IL-12p70 and IL-23 secretion was less marked than previously reported for monocytes. MyDCs produced lower concentrations of IL-23 than MoDCs, and no detectable IL-12p70.

It is known that *Hp* infection can have systemic effects, so next peripheral blood mononuclear cells (PBMCs) from 21 *Hp*+ and 13 uninfected patients were stimulated with *Hp* or control antigen and Th17 and Th1 cell frequencies analyzed by flow cytometry. A systemic *Hp*-specific Th17 response was identified with higher Th17 cell frequencies in the *Hp*+ patients compared to the uninfected controls (2.0-fold, p=0.027). A variable proportion of these cells also secreted IFNγ (median 33%, n=21), but there was no significant correlation between Th17 and Th1 cell frequencies. Peripheral blood Th1 cells were also increased in *Hp*+ patients (2.1-fold, p=0.018). No significant difference was found between peripheral blood Th17 and Th1 frequencies in the *Hp*+ patients.

The concentrations of Th17, Th1, Th2 and Treg cytokines in the gastric mucosa of *Hp*+ patients and uninfected controls were investigated using Luminex and real-time PCR. High levels of IL-17 expression in the infected compared to uninfected gastric mucosa were confirmed at both the mRNA and protein level (mRNA: 42.6-fold, $p<0.0001$; protein 3.5-fold, $p<0.0001$). IL-17 concentrations correlated with the levels of IL-17F ($\rho=0.80$, $p<0.0001$) and the chemokines CCL20 ($\rho=0.59$, $p<0.0001$) and IL-8 ($\rho=0.49$, $p=0.0004$). Concentrations of the Th17-differentiating cytokines IL-1β, IL-6, IL-21 and IL-23 were not increased in the *Hp*+ gastric biopsies, although IL-23 was present at high concentrations in all samples regardless of *Hp* infection status. IL-17 was present at higher concentrations than IFNγ (3.9-fold, $p<0.0001$), IL-4 (3.0-fold, $p<0.0001$) and IL-10 (6.8-fold, $p<0.0001$) in *Hp*+ gastric biopsies. *IFNG* mRNA was also more highly expressed than *IL17* mRNA (3.3-fold, $p=0.016$).

To identify the cellular source of the IL-17 mononuclear cells were extracted from gastric biopsies, stimulated with PMA/ionomycin and analyzed by flow cytometry. Amongst biopsy $CD3^+$ T cells from 10 *Hp*+ patients, IL-17 was produced mainly by $CD4^+$ Th17 cells (68.5%), although $CD8^+IL\text{-}17^+$ (24.7%) and $CD4^-CD8^-$ (18.2%) cells also made a significant contribution.

High IL-17 concentrations were associated with increased inflammation (2.4-fold, $p=0.024$) and neutrophil infiltration (2.4-fold, $p=0.031$). *RORC2* mRNA expression was weakly associated with PUD ($p=0.046$, 1.4-fold) but, surprisingly, no association was found between the IL-17 response and incidence of PUD or precancerous changes.

In conclusion *Hp* can stimulate DCs to produce IL-23 *in vitro*, and high levels of this Th17-differentiating cytokine were found in gastric mucosal biopsies. Stimulation with *dupA* null *Hp* strains led to reduced IL-12p70 and IL-23 secretion, suggesting a possible mechanism of action for this recently discovered virulence factor. Culturing *Hp* with MoDCs and MyDCs yielded quite different results, and it remains unknown whether either model closely reflects gastric mucosal DC responses. *Hp*-specific Th17 responses were

identified in the peripheral blood of patients with active *Hp* infection for the first time. Th17 cells were identified as the main cellular source of IL-17 in the *Hp*-infected gastric mucosa but there were also significant numbers of $CD8^{+}IL\text{-}17^{+}$ and $CD4^{-}CD8^{-}IL\text{-}17^{+}$ T cells, which have not previously been described in this context. High mucosal concentrations of IL-17 and association of this cytokine with infiltration of immune cells indicate that it is an important component of the human immune response to *Hp*. However, no association between IL-17 and risk of disease was detected in this study, although *RORC2* expression was weakly associated with PUD. The role of IL-17/Th17 in Hp-related disease warrants further investigation.

LIST OF PUBLICATIONS AND PRESENTATIONS

Publications

Staples, E., Ingram, R. J. M., Atherton, J. C., Robinson, K. **Optimising the quantification of cytokines present at low concentrations in small human mucosal tissue samples using Luminex assays.** Submitted to the Journal of Immunological Methods.

Oral Presentations

Staples, E., Ingram, R. J. M., Hussain, K., Letley, D. P., Atherton, J. C., Robinson, K. **Th17 cells are increased in *Helicobacter pylori* infection and may be associated with peptic ulcer disease.** Digestive Diseases Federation, Liverpool, 17-20th June 2012. (Delivered by R. J. M. Ingram)

Letley, D. P., Hussain, K., **Staples**, E., Robins, R. A., Ghaemmaghami, A. M., Shakib, F., Atherton, J. C., Martinez-Pomares, L., Robinson, K. ***Helicobacter pylori*-induced protection from allergy is associated with peripheral blood regulatory T cells.** BSI: Regulatory T cells in Inflammatory and Infectious Diseases: New Horizons for Old Friends, UCL Institute of Child Health, 5th March 2010. (Delivered by E. Staples)

**Won Best Abstract Prize: A years subscription to Nature Reviews Immunology*

Poster Presentations

Staples, E., Ingram, R. J. M., Marx, C., Wang, Q., Franks, H., Hussain, K, Letley, D. P., Jackson, A., Atherton, J. C., Robinson, K. **Are Th17 or Th1 cells dominant in human *Helicobacter pylori* infection?** 10th International Workshop on Pathogenesis and Host Response in *Helicobacter* infections. Konventum, Helsingor, Denmark, 4-7th July 2012.

Staples, E., Ingram, R. J. M., Hussain, K., Letley, D. P., Atherton J. C., Robinson, K. **Do Th17 or Th1 cells dominate the immune response to human *Helicobacter pylori* infection?** Regulatory T Cells & T Helper Cells Symposium, Royal Vetinary College, University of London, 30th March 2012

Staples, E., Ingram, R. J. M., Hussain, K., Letley, D. P., Atherton J. C., Robinson, K. **Th17 cells are increased in *Helicobacter pylori* infection and may be associated with peptic ulcer disease.** The Royal Society of Medicine: Clinical Immunology and Allergy Section, Spotlight on Infectious Diseases Meeting, Royal Society of Medicine, 10-11th February 2012.

Staples, E., Patel, S. R., Kenefeck, R. M. W., Hussain, K., Letley, D. P., Atherton, J. C., Robinson, K. ***Helicobacter pylori* – induced regulatory T cells help evade immune clearance but reduce pathology.** Society for General Microbiology Spring Meeting, Edinburgh, April 2010.

ACKNOWLEDGEMENTS

My supervisors Dr Karen Robinson and Professor John Atherton for their support, guidance and encouragement.

The rest of *Helicobacter pylori* research group, particularly Dr Richard Ingram, Dr Darren Letley, Dr Khiyam Hussain, Dr Christian Marx, Katie Cook, Helle Skjoldmose, Benoit Biousse and the Nottingham Digestive Diseases Biomedical Research Unit support technicians Mel Lingaya and Rav Banwait.

Dr Hester Franks, Qunwei Wang, Andrew Jackson, Dr Ian Spendlove, Academic Unit of Clinical Oncology, University of Nottingham.

Dr Abed Zaitoun, Department of Histopathology, Queen's Medical Centre, Nottingham University Hospitals.

Dr Ann Lowe and Professor Jan Bradley, Department of Biology, University of Nottingham.

Professor John Atherton, Barbara Pick, the other endoscopists and all the patients who donated samples at Nottingham University Hospitals.

The Medical Research Council.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS USED

AhR	Aryl hydrocarbon receptor
BMDC	Bone marrow-derived dendritic cell
CagA	Cytotoxin associated gene A
*cag*PAI	*cytotoxin associated gene* pathogenicity island
CFU	Colony forming units
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DupA	Duodenal ulcer promoting gene
EAE	Experimental autoimmune encephalomyelitis
ECL	Enterochromaffin-like
ELISA	Enzyme-linked immunosorbent assay
FOXP3	Forkhead box protein 3
Hp	*Helicobacter pylori*
IFNγ	Interferon-gamma
IL-17	Interleukin-17
ILC	Innate lymphoid cell
ILC3	IL-17- and/or IL-22-producing innate lymphoid cell
IM	Intestinal metaplasia
LPS	Lipopolysaccharide
LTi	Lymphoid tissue-inducer
MHC	Major histocompatability complex
MIF	Macrophage migration inhibitory factor
MoDC	Monocyte-derived dendritic cell
MOI	Multiplicity of infection

MyDC	CD1c+ myeloid dendritic cell
NCR	Natural cytotoxicity triggering receptor
NOD	Nucleotide-binding oligomerization domain
ns	Not significant
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PPI	Proton pump inhibitor
PRR	Pattern recognition receptor
PUD	Peptic ulcer disease
ROR	Retinoic acid receptor–related orphan receptor
T-bet	T-box expressed in T cells
TBX21	T-box protein 21 (gene encoding T-bet)
Tc	T cytotoxic ($CD8^+$)
TCR	T cell receptor
Th	T helper ($CD4^+$)
TLR	Toll-like receptor
TNFα	Tumour necrosis factor-alpha
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
Wt	Wild-type

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 *HELICOBACTER PYLORI* INFECTION AND DISEASE

1.1.1 *Helicobacter pylori*: The Bacteria

Helicobacter pylori (*Hp*) is a Gram negative spiral shaped bacterium. The bacterium and its link to peptic ulcer disease were discovered by Warren (a pathologist) and Marshall (a clinical fellow at the time) (Marshall and Warren, 1984). Prior to Warren and Marshall's publication it was thought very unlikely that any infecting microorganisms could survive in the harsh acid environment of the stomach and peptic ulcer disease was thought to be largely due to stress and lifestyle factors. Marshall infected himself with *Hp* to demonstrate that it caused gastritis, which resolved with antibiotic treatment, thereby fulfilling Koch's postulates. Testing for *Hp* and, if found, eradicating with a triple drug therapy (consisting of two antibiotics and a proton pump inhibitor (PPI)) is now the most common first line treatment for peptic ulcer disease (Wolle and Malfertheiner, 2007). Marshall and Warren were awarded the Nobel Prize in 2005 for their seminal findings (Nobelprize.org, 2005).

Evidence suggests that *Hp* has colonized the human stomach and co-evolved with its human host for at least 50,000 years (Atherton and Blaser, 2009). Point mutation and intragenomic and intergenomic recombination result in genetic diversity and enable *Hp* to adapt to its harsh acidic niche in the stomach (Atherton and Blaser, 2009). In order to survive *Hp* expresses large amounts of urease enzyme, which converts urea to ammonia and carbon dioxide, buffering the bacterium from the acid environment of the stomach (Dunn et al., 1990). This reaction also forms the basis of some of the tests used to detect *Hp* infection including the urea breath test, and the biopsy urease test used in this study (Dunn et al., 1990, Ricci et al., 2007). *Hp* also has characteristic flagellae (2-6 per bacterium), which allow the bacteria to move through the mucus (Dunn et al., 1997, Suerbaum et al., 1993). Motility is essential for robust infection of the gastric mucosa (Ottemann and Lowenthal, 2002), presumably allowing the bacteria to rapidly swim from the acidic

stomach lumen into the thick mucus layer which protects the gastric epithelium.

Following its isolation *Hp* was initially named *Campylobacter pyloridis.* This was corrected to *Campylobacter pylori,* prior to *Helicobacter* becoming a separate genus in 1989 (Goodwin et al., 1989). Virtually all mammals, including dolphins and whales, have been found to be colonized by *Helicobacter* species, most of which colonize the intestine rather than the stomach (Owen, 1998, Agency, 2011, Goodwin et al., 1989). *Hp* is the main human pathogen but *H. cinaedi, H. canis, H. pullorum* and *H. fenelliae* have also been reported to cause human infection (Agency, 2011). Serum antibodies to non-gastric *Helicobacter* species have been reported to be more prevalent in patient with autoimmune liver disease (Nilsson et al., 2003). Other *Helicobacter* species that infect the gastric mucosa include *H. felis,* which can infect cats, dogs and mice, *H. mustelae* which infects ferrets and *H. suis* which infects pigs (Owen, 1998). *H. felis* can cause the development of preneoplastic lesions in C57BL/6 mice and lymphoma in BALB/c mice (Sakagami et al., 1996). Until recently *Hp* strains that can colonize mice and recapitulate pathology similar to that seen in *Hp*-infected humans have not been available, so the *H. felis* mouse infection model has been widely used (Pritchard and Przemeck, 2004, Sayi et al., 2009, Roth et al., 1999, Hitzler et al., 2012a). The immune response to *Helicobacter hepaticus,* a murine intestinal pathogen has also been well studied and is employed as a model for inflammatory bowel disease (Rao et al., 2006, Kullberg et al., 2006, Buonocore et al., 2010, Mazmanian et al., 2008).

1.1.2 Epidemiology and Transmission

Hp is one of the commonest bacterial infections in the world (Ezra J. Barzilay and Fagan, 2012). Its estimated prevalence is 70% in developing countries and 30-40% in industrialized countries, though estimates vary widely depending on the study population and methods used (Ezra J. Barzilay and Fagan, 2012, Bruce and Maaroos, 2008, Goh et al., 2011, Dunn et al., 1997). The infection is usually acquired in early childhood and persists life-long in the mucus layer of

the stomach unless treated (Bruce and Maaroos, 2008, Robinson et al., 2007). Transmission requires close personal contact and is thought to be via the oral-oral route (Goh et al., 2011). *Hp* causes chronic gastritis, but this is asymptomatic in the vast majority of those infected, only causing disease in about 10-15% of cases (Robinson et al., 2007, Atherton, 2006). A number of factors can influence the risk of disease including the host immune response, which is the focus of this study. Bacterial virulence factors and environmental factors, also contribute to disease risk, as discussed below.

1.1.3 *Helicobacter pylori*-Related Disease

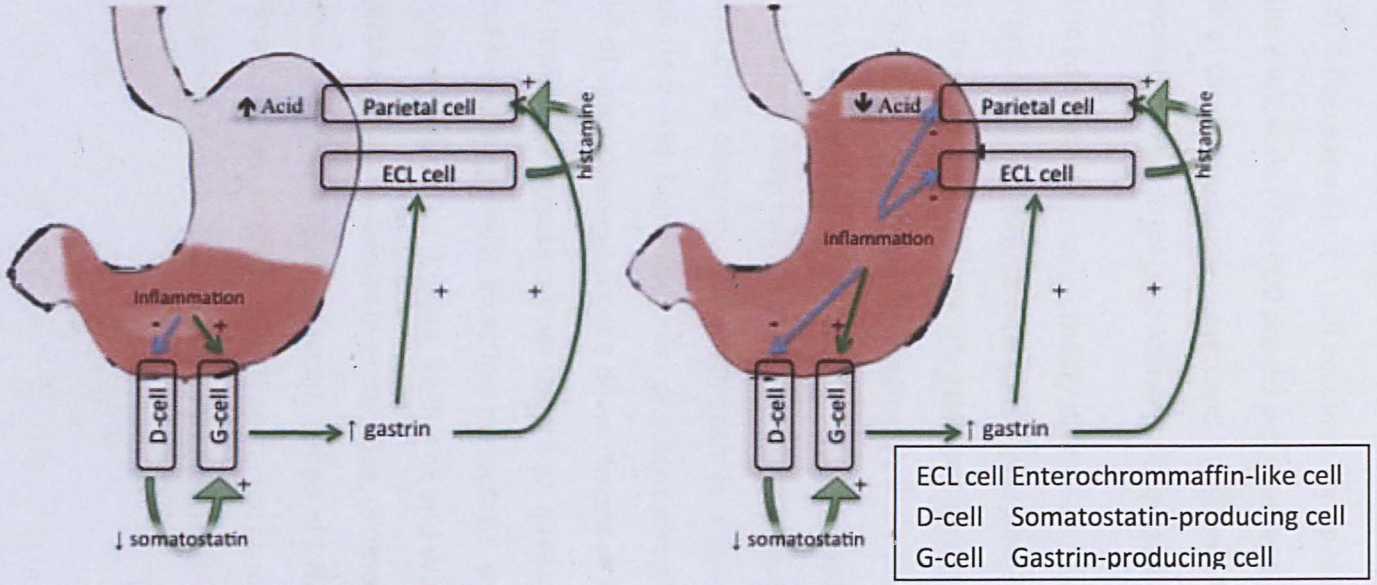
1.1.3.1 Peptic ulcer disease

Hp is the major cause of both gastric and duodenal ulcers. Peptic ulcer disease causes considerable morbidity and the complications of haemorrhage and perforation cause significant mortality, especially in the elderly and those with severe comorbidity (Alakkari et al., 2011, Rockall et al., 1995). *Hp*-associated ulceration is associated with inflammation and causes characteristic epithelial damage (Chan et al., 1991, Atherton, 2006). Eradication of the bacteria can heal ulcers and prevents their recurrence (Atherton, 2006).

The pattern of colonization in the stomach can influence which disease develops (Figure 1.1). Antral-predominant colonization leads to increased gastrin secretion by G-cells, which stimulates acid production from the parietal cells in the oxyntic glands of the healthy corpus (Robinson and Atherton, 2009). This is mediated both by a direct effect and by stimulation of histamine release from enterochromaffin-like (ECL) cells (Zavros and Merchant, 2005). Antral inflammation also reduces somatostatin production by D-cells. Somatostatin provides negative feedback to G-cells, so reduction in somatostatin leads to hypergastrinaemia. The high stimulated gastric acid output can lead to gastric metaplasia of the duodenum, allowing *Hp* to colonize and potentially cause duodenal ulceration (Robinson and Atherton, 2009). When *Hp* colonization is corpus-predominant or pangastric there is

usually normal or reduced acid production, despite stimulation of G-cells (Atherton, 2006). This is due to inhibition of acid secretion by pro-inflammatory mediators such as IL-1β and TNFα. It has been suggested that *Hp* is not normally able to colonize the corpus unless acid secretion is reduced (Marshall and Warren, 1984). Chronic hypergastrinaemia can lead to atrophy of the acid-secreting glands, causing further gastrin secretion and setting up a positive feedback loop.

Duodenal ulcer patients have a reduced risk of gastric cancer compared to gastric ulcer patients (Hansson et al., 1996). Non-steroidal anti-inflammatory drugs are the other major cause of peptic ulcer disease.



ANTRAL –PREDOMINANT INFLAMMATION

Results in:	Increased stimulated gastric acid output
Leads to:	Gastric metaplasia of duodenum
	Hp colonization of duodenum
	Increased risk of duodenal ulceration

PANGASTRIC INFLAMMATION

Results in:	Reduced gastric acid output
Leads to:	Colonization by other bacteria and fungi
	Hypergastrinaemia
	Gastric gland atrophy
	Increased risk of gastric ulceration
	Increased risk of gastric adenocarcinoma

Figure 1.1 Patterns of *Hp* colonization, their effects on acid production and associations with clinical disease. Adapted from (Robinson and Atherton, 2009).

1.1.3.2 Gastric adenocarcinoma

In 1994 *Hp* was the first bacterium to be classified as a carcinogen by the World Health Organization (WHO) (IARC, 1994). The incidence of gastric cancer is decreasing but it still accounts for approximately 10% of all cancer deaths worldwide (738,000 deaths per year) and is the 3rd most common cause of cancer mortality in men and the 5th in women (Jemal et al., 2011). *Hp* is associated with distal (also known as non-cardia) gastric adenocarcinoma.

Gastric adenocarcinoma is broadly divided into intestinal-type (well differentiated) and diffuse-type (undifferentiated), as proposed by Lauren in 1965, though further subclassification and categorization of rare histological variants in an "interderminate" category were proposed by the WHO in 2010 (Lauren, 1965, Hu et al., 2012). The intestinal-type is the commonest and is most strongly associated with *Hp* infection (Hu et al., 2012, Parsonnet et al., 1991). Correa observed that gastric cancer occurred more frequently in populations with high incidences of gastric atrophy and proposed a multistep model of carcinogenesis for the development of intestinal-type gastric cancer, with stepwise progression from superficial gastritis to atrophic gastritis (loss of acid-secreting glands), intestinal metaplasia, dysplasia and finally carcinoma (Figure 1.2) (Correa, 1988, Fox and Wang, 2007). Progression along this pathway usually occurs over decades, promoted by *Hp*-induced chronic inflammation which leads to accumulation of DNA damage and mutations. *Hp* also increases the risk of diffuse type gastric cancer but the histological development of this subtype is less well defined.

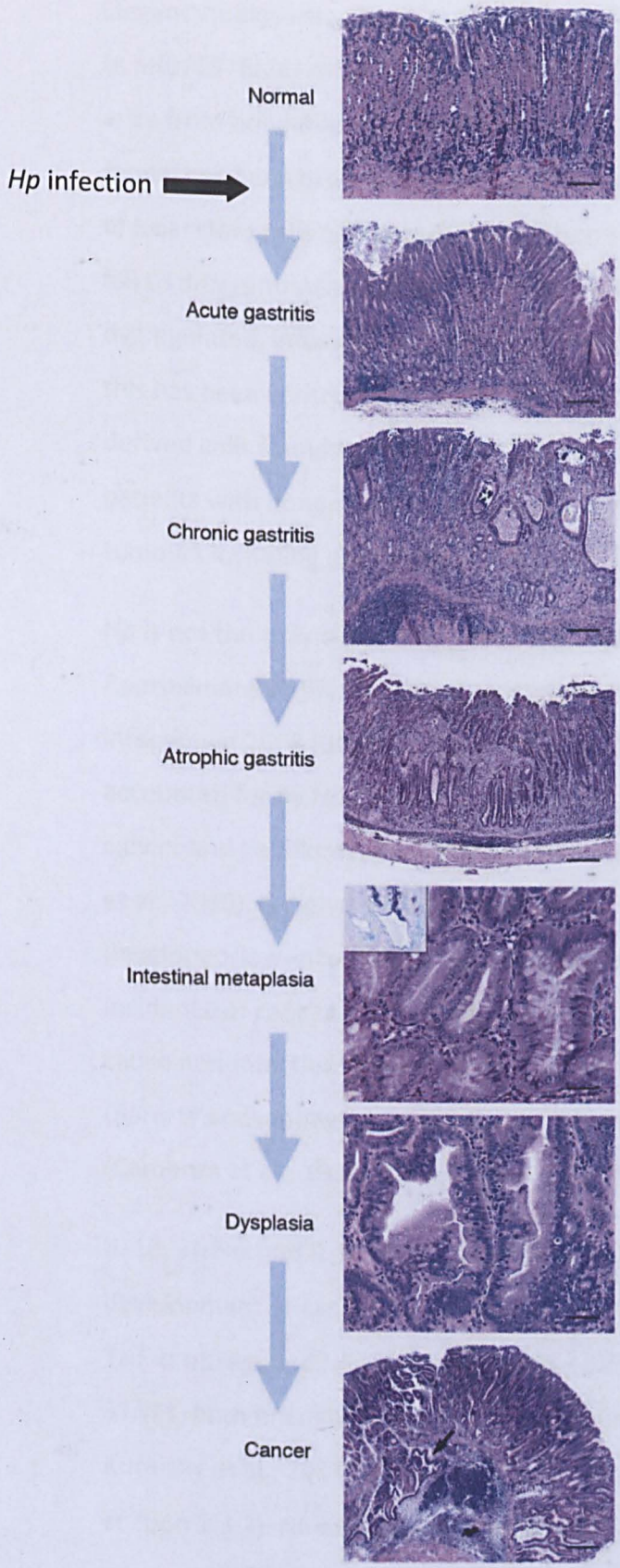


Figure 1.2 Correa multistep model of the development of intestinal-type gastric adenocarcinoma. (From Fox *et al.* (Fox and Wang, 2007)).

Elegant studies using bone marrow transplants from transgenic mice into the *H. felis*/C57BL/6J mouse model of *Hp* have shown that gastric cancer may arise from bone marrow derived stem cells (Houghton and Wang, 2005). A model has been proposed whereby chronic inflammation leads to exhaustion of local stem cells and recruitment of bone marrow derived stem cells which fail to differentiate and become metaplastic, dysplastic then cancerous in the dysregulated, inflamed gastric tissue (Houghton and Wang, 2005). However, this has been controversial and is not supported by the lack of bone marrow-derived cells found in the neoplastic epithelium of patients in a study of patients with bone marrow transplants who had developed solid organ tumours including gastric cancer (Worthley et al., 2009).

Hp is not the only chronic infection associated with development of cancer. Approximately 16% (2 million) cancers worldwide were attributable to infection in 2008 (de Martel et al., 2012). The vast majority of these were accounted for by *Hp*-related gastric cancer, hepatitis B- and C-related liver cancer and papillomavirus-related cervical cancer (de Martel et al., 2012, Ott et al., 2010). A higher proportion of cancer deaths was due to infection in less developed/low-income countries (de Martel et al., 2012, Ott et al., 2010). The incidence of cancer is also increased in gastrointestinal conditions which cause non-infection related chronic inflammation such as reflux oesophagitis (Barrett's oesophagus), inflammatory bowel disease and coeliac disease (Cameron et al., 1985, Itzkowitz and Yio, 2004, West et al., 2004).

IL-1β, TNF-α and IL-6 are key pro-inflammatory cytokines involved in the development of cancer (El-Omar et al., 2000, Chiba et al., 2012). IL-1β and TNF-α upregulate NF-κB expression in epithelial cells and IL-6 signals via STAT3: both oncogenic transcription factors (Ben-Neriah and Karin, 2011, Kuraishy et al., 2011). STAT3 signalling promotes Th17 differentiation (see section 1.3.2). NF-κB has a number of pro-tumorigenic effects including promotion of tumour cell growth and survival and stimulation of growth factors, cytokines, cyclooxygenase-2 (COX-2) and reactive oxygen species (Ben-Neriah and Karin, 2011). Reactive oxygen species and mitogen-activated

protein kinase (MAPK) cascades triggered by cytokines can activate proto-oncogenes such as c-Fos, c-Jun and c-Myc (Hussain et al., 2003). The *Hp* virulence factor cytotoxin associated gene A (CagA) can bind SRC- homology 2 domain containing tyrosine phosphatase (SHP-2), thereby directly influencing receptor kinase signalling (Higashi et al., 2002). *Hp* also upregulates epidermal growth factor expression in a CagA-dependent manner *in vitro* (Keates et al., 2007).

Atrophy is a loss of acid-producing glands and IL-1β and TNF-α also directly suppress acid production by parietal cells (Atherton, 2006). Some *Hp* antigens are similar in structure to the gastric proton pump ATPase which can lead to autoimmune destruction of the pump, further reducing acid secretion (Amedei et al., 2003, Kiriya et al., 2007). The increased gastric pH may allow other pathogens to infect the stomach, further adding to the inflammation. Gastrin is produced in order to stimulate acid production in the face of the increased pH. IL-8, IL-1β and TNFα can also stimulate gastrin production which promotes epithelial cell proliferation, NF-κB activation and COX-2 expression (Robinson et al., 2007). Atrophy is associated with loss of the morphogen sonic hedgehog, which may have a role in early neoplastic transformation (Shiotani et al., 2005).

Hp can induce apoptosis, however mediators such as COX-2, which catalyzes production of prostaglandin-E2, and nitric oxide that are upregulated during *Hp* infection are anti-apoptotic (Zhang et al., 2007, Robinson et al., 2007). Prolonged survival of damaged cells could allow mutations to accumulate. Houghton *et al.* used a mouse transplant model and found that bone marrow-derived stem cells were recruited to in the context of chronic inflammation and could give rise to gastric cancer (Houghton and Wang, 2005). This is proposed to be due to local tissue stem cell failure following prolonged increased cell turnover, but the new stem cells themselves will, in turn, be subject to the local *Hp*-induced inflammation.

In addition to the increased reactive oxygen species mediated via *Hp*-induced cytokines, *Hp* can also directly stimulate generation of DNA-damaging reactive oxygen species (Obst et al., 2000). Recruitment and activation of phagocytes and other pro-inflammatory cells, by IL-17 signalling and other mechanisms, will further boost levels of oxygen and nitrogen radicals and oxidative stress. IL-17 also increases inducible nitric oxide synthase expression (Miljkovic and Trajkovic, 2004). Expression of the RNA- and DNA-editing enzyme activation-induced cytidine deaminase (AID), which has a physiological role in generation of antibody diversity via somatic hypermutation and class switch recombination, is aberrant in *Hp*-infected gastric cell lines and in a number of malignancies (Matsumoto et al., 2007, Chiba et al., 2012). *Hp* also reduces levels of mismatch repair proteins, which is likely to increase the probability of mutations accumulating (Kim et al., 2002).

Epigenetic changes can also influence expression of tumour suppressor and oncogenes. *Hp* causes aberrant DNA methylation, which has been linked to carcinogenesis (Maekita et al., 2006, Nakajima et al., 2009, Bae et al.). Recent studies have raised the possibility that methylation patterns in specific genes/DNA elements could act as prognostic markers (Bae et al., Nakajima et al., 2009). MicroRNAs are small, non-coding RNAs that can regulate gene expression post-transcriptionally. The effects of these on *Hp*-induced inflammation and gastric cancer have not been widely studied, but microRNA-155 was upregulated in response to *Hp* and appeared to have a negative effect on *Hp*-induced inflammation (Xiao et al., 2009).

Following malignant transformation, new vessels are required to support the growing tumour. Cytokines induced by *Hp* infection including IL-8, IL-1β, IL-6 and IL-17 are pro-angiogenic and reactive oxygen species and nitrogen oxide can also promote angiogenesis (Robinson et al., 2007, Numasaki et al., 2003). Metalloproteinases may facilitate tumour invasion and metastasis and a number of these can be upregulated by IL-17 receptor stimulation (MMP1,MMP3, MMP9, MMP13) (Gaffen, 2008).

Tregs, which are increased during *Hp* infection, may inhibit anti-tumour immunosurveillance. After malignant transformation cancerous cells can promote expansion of Tregs (Ghiringhelli et al., 2006). Tregs can inhibit NK cells, $CD8^+$ T cells and $CD4^+$ T cells with anti-tumour activity (Ghiringhelli et al., 2006, Wang and Wang, 2005, Zhou and Levitsky, 2007).

1.1.3.3 Gastric Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma

Hp is the main cause of B cell MALT lymphoma, which can transform to high-grade diffuse large-cell B cell lymphoma. Ongoing antigenic stimulation appears to play a role in clonal expansion (Du et al., 1996). Like gastric adenocarcinoma, MALT lymphoma is associated with a pan-gastric pattern of inflammation (Miehlke et al., 2001). Guidelines recommend *Hp* eradication, which leads to regression in over 70% patients when treated early, however relapse can occur so long-term follow up is recommended (Wotherspoon et al., 1993, Zullo et al., 2010). Mucosa-associated lymphoid tissue does not occur in the uninfected stomach.

1.1.3.4 Other Hp-related disease

Hp infection has also been associated with a number of extra-gastric diseases but, as it is common, studies need to be well designed to avoid making spurious connections. The best evidence is for idiopathic thrombocytopenic purpura (ITP) and unexplained iron deficiency anaemia (Tan and Goh, 2012, Pellicano et al., 2009). In a small randomized trial and systematic reviews *Hp* eradication led to increases in platelets in up to 50% *Hp*+ patients with ITP, especially those with mild disease (Suzuki et al., 2005, Stasi et al., 2009, Arnold et al., 2009). Suzuki *et al.* found higher titres of antibodies to the virulence factor CagA in the serum of those that responded to *Hp* eradication (Suzuki et al., 2005). This is consistent with suggestions that ITP in *Hp*+ patients may be due to cross-reactivity between platelet-associated immunoglobulin G and anti-CagA antibodies (Takahashi et al., 2004). International guidelines recommend *Hp* eradication in patients with ITP (Fock et al., 2009, Malfertheiner et al., 2012).

Unexplained iron deficiency anaemia (IDA) is more common in *Hp*+ patients, though it only affects a small proportion of *Hp*+ patients (Tan and Goh, 2012). The underlying mechanism is not clear but may relate to impaired iron absorption during *Hp* infection (Tan and Goh, 2012). A meta-analysis of 15 observational studies confirmed an association between *Hp* infection and IDA (Qu et al., 2010). In five randomized controlled trials analyzed in the same study *Hp* eradication led to trends of increases in haemoglobin (Hb) and serum ferritin levels, but the changes did not reach statistical significance (Qu et al., 2010). Larger meta-analyses found that *Hp*+ patients managed with *Hp* eradication and oral iron achieved greater increases in Hb and serum ferritin than those treated with oral iron only (Huang et al., 2010, Yuan et al., 2010). Guidelines recommend *Hp* eradication in infected patients with unexplained IDA (Fock et al., 2009, Malfertheiner et al., 2012).

Other diseases that have been linked to *Hp* include ischaemic heart disease, ischaemic stroke, parkinson's disease, alzheimer's disease, obesity (associated with changes in leptin and ghrelin levels) and chronic idiopathic urticaria. Evidence associating these diseases with *Hp* is not compelling with conflicting data for some and small or uncontrolled studies for others (Pellicano et al., 2009, Tan and Goh, 2012).

1.1.3.5 Diseases where Hp may have a protective role

Epidemiological studies show a negative association between gastro-oesophageal reflux disease (GORD) and *Hp* (O'Connor, 1999). The prevalence of GORD is increasing, whilst the prevalence of *Hp* infection is falling. GORD can lead to metaplasia, whereby the epithelium of the lower oesophagus which is normally squamous, becomes columnar (Barrett's oesophagus). This in turn predisposes to the development of adenocarcinoma. These sequelae of GORD are also less common in those with *Hp* infection (Rokkas et al., 2007). However, there is no evidence that eradicating *Hp* causes worsening of GORD symptoms (Malfertheiner et al., 2012). A routine "test and treat" strategy is not recommended for patients with GORD, however it is recommended that testing is considered in those on long-term proton pump

inhibitors (PPI), which are widely used to treat GORD (Fock et al., 2009). This is because *Hp*+ patients treated with long-term PPIs tend to develop corpus-predominant inflammation with atrophy (Malfertheiner et al., 2012).

The incidence of allergic diseases in industrialized countries has been increasing, while the prevalence of *Hp* infection has been falling (Chen and Blaser, 2007). The "hygiene hypothesis" was initially proposed by Strachan in 1989, who found that hayfever was inversely related to the number of children in the household (Strachan, 1989). This hypothesis has been controversial. Initially it was suggested that infections early in childhood would cause a Th1 bias, therefore shifting the Th1/Th2 balance away from Th2-allergy causing $CD4^+$ T cells. More recently it has been suggested that infections early in life are associated with increased Treg responses, which could provide an explanation for increases in autoimmune disease, in addition to allergy (Rook, 2007).

Blaser *et al.* reviewed 12 cross-sectional and 4 case-control studies investigating the relationship of *Hp* with asthma and other allergic diseases. Most of the cross-sectional studies showed significant inverse association between *Hp* and allergy, whereas the case-control studies, which were generally smaller, did not. A larger study by Chen *et al.* found that *Hp* infection was inversely associated with "allergy symptoms"(Chen and Blaser, 2007). Inverse associations between *Hp* and a number of other markers of allergy reached statistical significance for the younger subgroup and the group with CagA+ *Hp* (Chen and Blaser, 2007). A study of 3 year old Ethiopian children found a reduced risk of eczema in those with *Hp* infection (Amberbir et al., 2011).

There is now some experimental evidence from mouse models of allergy demonstrating a protective role for *Hp*. Arnold *et al.* showed that *Hp* protected against asthma, as measured by airway hyperreactivity, tissue inflammation and goblet cell hyperplasia, in airway inflammation induced by ovalbumin or house dust mite (Arnold et al., 2011a). Protection was most

robust in mice infected with *Hp* neonatally and appeared to be Treg mediated (Arnold et al., 2011a). My collegues have also investigated the effects of *Hp* infection on Der p 1 (immunodominant house dust mite allergen) sensitization and found that *Hp*-infected mice had lower levels of total and Der p 1-specific IgE, and less macrophage and lymphocyte infiltration (Hussain, 2012). *Hp*-mediated protection from airway hyperresponsiveness and inflammation seems to depend on DC-derived IL-18, which drives Treg differentiation (Oertli et al., 2012).

Serology-based studies have found reduced rates of *Hp* infection amongst patients with multiple sclerosis. Preliminary experiments suggest that *Hp* protects against experimental autoimmune encephalomyelitis (EAE), a well studied mouse model of central nervous system inflammation (Hussain, 2012).

1.1.4 Diagnosis and Treatment of *Hp*

1.1.4.1 Diagnostic tests for Hp

Non-invasive tests for *Hp* include the urea breath test (based on *Hp*'s urease activity) and stool antigen testing (Malfertheiner et al., 2012). Both these tests indicate current infection. Serology testing is also widely available, but can stay positive for some time following *Hp* eradication. It is suggested that a positive serology result is followed up by one of the non-invasive tests above or endoscopy (Ezra J. Barzilay and Fagan, 2012). Upper gastrointestinal endoscopy allows inspection of the oesophagus, stomach and upper duodenum, and biopsies can be taken for urease test, culture and sensitivities, and histology.

A "test and treat" strategy, using non-invasive testing, is advocated for younger patients with dyspepsia and no "alarm" symptoms (weight loss, dysphagia, gastrointestinal bleeding, abdominal mass and iron deficiency anaemia, which are associated with increased risk of gastric cancer) (Malfertheiner et al., 2012, Fock et al., 2009). Older patients and those with "alarm" symptoms should be referred for endoscopy.

The Updated Sydney Scoring System is the most widely used scheme for histopathological assessment of gastritis. Five variables (*Hp* density, neutrophil infiltration, lymphocyte infiltration, atrophy and intestinal metaplasia) are graded as normal (0), mild (1), moderate (2) or marked (3) (Figure 1.3)(Dixon et al., 1996). An experienced gastrointestinal histopathologist is required.

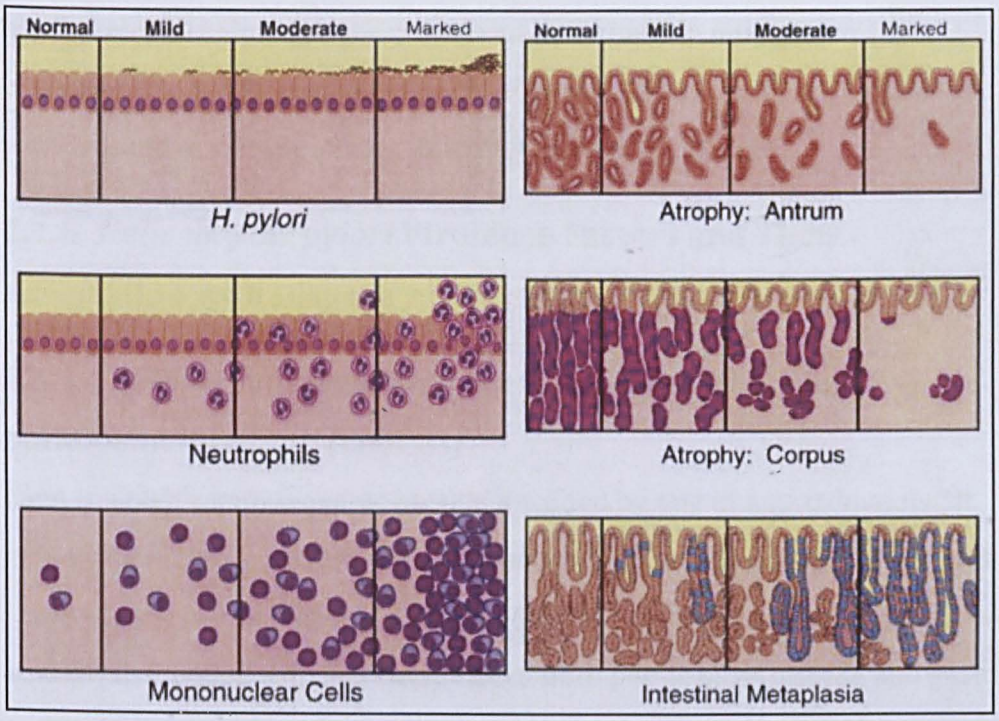


Figure 1.3 The Updated Sydney Scoring System. Schematic representation of variables to aid grading. Taken from (Dixon et al., 1996).

1.1.4.2 Treatment of Hp

A 7 or 14 day course of triple therapy with a PPI, amoxicillin and clarithromycin or metronidazole have been standard treatment for *Hp* (Fock et al., 2009, Malfertheiner et al., 2007). However, increasing antibiotic resistance, particularly to clarithromycin, is resulting in higher rates of eradication failure (Malfertheiner et al., 2012, Graham and Gisbert, 2013). A number of different strategies have been suggested, including bismuth quadruple therapy (PPI, bismuth, tetracycline and metronidazole). >95%

eradication rates have been achieved with sequential therapies, such as PPI and amoxicillin for 14 days with clarithromycin and metronidazole added for the final 7 days, and PPI and amoxicillin for 10 days with addition of rifabutin and ciprofloxacin on days 6-10, but these regimens are not yet recommended for widespread use (Hsu et al., 2011, Tay et al., 2012, Graham and Gisbert, 2013). Treatment should be guided by local antibiotic resistance patterns, and in resistant cases by antibiotic sensitivities of the *Hp* strain infecting the individual, if possible. A number of patient factors can influence success of eradication, including genetic polymorphisms that can influence bioavailability of PPI and gastric acidity. Smoking and obesity are associated with reduced eradication rates (Malfertheiner et al., 2012).

1.1.5 *Helicobacter pylori* Virulence Factors and Their Association with Disease

1.1.5.1 Cytotoxin associated gene A (CagA) and the cag *pathogenicity island (cag*PAI*)*

CagA is a highly immunogenic protein encoded by one of approximately 30 genes that make up the *cag* (*cytotoxin-associated gene*) pathogenicity island (*cag*PAI), and is often used as a marker for the island. *cagA*+ strains are strongly associated with increased risk of both peptic ulcer disease and gastric cancer (Atherton, 2006).

The *cag*PAI encodes a type IV secretion system, which acts as a molecular syringe, injecting bacterial proteins, including CagA, into gastric epithelial cells. Once inside the host cell, CagA tyrosine phosphorylation motifs are phosphorylated by Src family kinases. CagA can activate transcription factors and oncogenes including SHP-2, c-fos, c-jun and c-met with effects on multiple cellular processes including migration, proliferation and apoptosis (Figure 1.4)(Blaser and Atherton, 2004, Atherton, 2006, Higashi et al., 2002).

CagA has phosphorylation sites which consist of the amino acid motif EPIYA. These motifs are classified as type A, B, C or D, depending on the subsequent amino acid sequence. The A and B motifs are found in strains from all over the

world, but the C motif is predominantly found in *Hp* strains from Western countries and the D motif in strains from East Asia (Argent et al., 2008, Polk and Peek, 2010). The D motif interacts strongly with SHP-2, which may partly explain the high incidence of gastric carcinoma found in East Asia (Azuma et al., 2004). The D motif is rarely duplicated but multiple C motifs can occur (Argent et al., 2008). Strains with more tyrosine phosphorylation motifs induce more cytoskeletal changes and are more closely associated with gastric cancer (Argent et al., 2004, Ferreira et al., 2012).

Some effects of CagA are independent of tyrosine phosphoylation. For example, it can bind tight junction proteins such as ZO-1, leading to loss of epithelial barrier polarity and integrity (Amieva et al., 2003). The *cag*PAI type IV secretion apparatus also allows delivery of peptidoglycan into host cells where it binds the intracellular pattern recognition receptor NOD1 which signals through NF-κB, activating pro-inflammatory genes, including IL-8 (Figure 1.4)(Viala et al., 2004). IL-8 is a chemokine and has a key role in attracting neutrophils to the *Hp*-infected stomach. Increased neutrophil infiltration induced by *cagA*+ strains may contribute to their increased pathogenicity (Yamaoka et al., 1998).

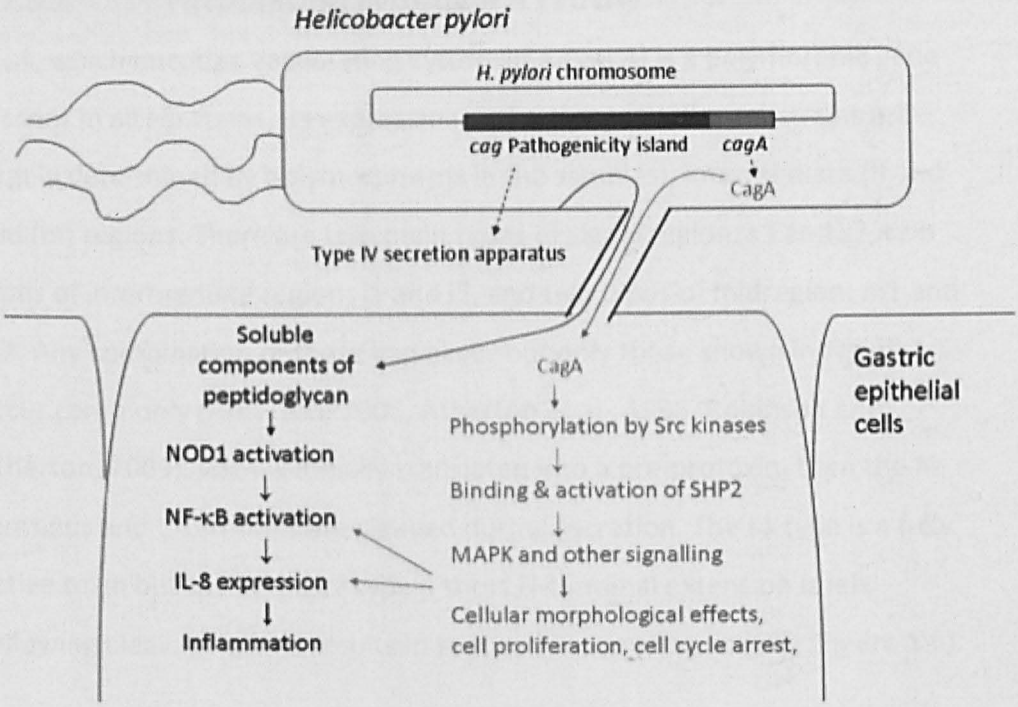


Figure 1.4 Host signalling events induced by the *cag* pathogenicity island (taken from(Robinson and Atherton, 2009)). CagA translocated into gastric epithelial cells via type IV secretion apparatus encoded by the *cag*PAI is phosphorylated by Src kinase and activated SHP-2 and MAPK signalling cascades leading to effects on multiple cellular processes including proliferation, motility and apoptosis. The type IV secretion system also translocates peptidoglycan which binds NOD1 leading to NF-κB stimulation and thereby upregulating expression of pro-inflammatory genes including IL-8.

1.1.5.2 *Vacuolating cytotoxin A (VacA)*

vacA, which encodes Vacuolating cytotoxin A (VacA) is a polymorphic gene present in all *Hp* stains. Its expression and activity in different strains are largely determined by polymorphisms in the signal (s), intermediate (i) and mid (m) regions. There are two main types of signal region: s1 and s2, two types of intermediate region: i1 and i2, and two types of midregion: m1 and m2. Any combination of these can occur but only those shown in Figure 1.5 occur commonly (Atherton, 2006, Atherton et al., 1995, Robinson and Atherton, 2009). *vacA* is initially translated into a pre-protoxin, then the N-terminus and C-terminus are cleaved during secretion. The s1 type is a fully active toxin but the in the s2 type a short N-terminal extension is left following cleavage which results in reduced vacuolating activity (Figure 1.5).

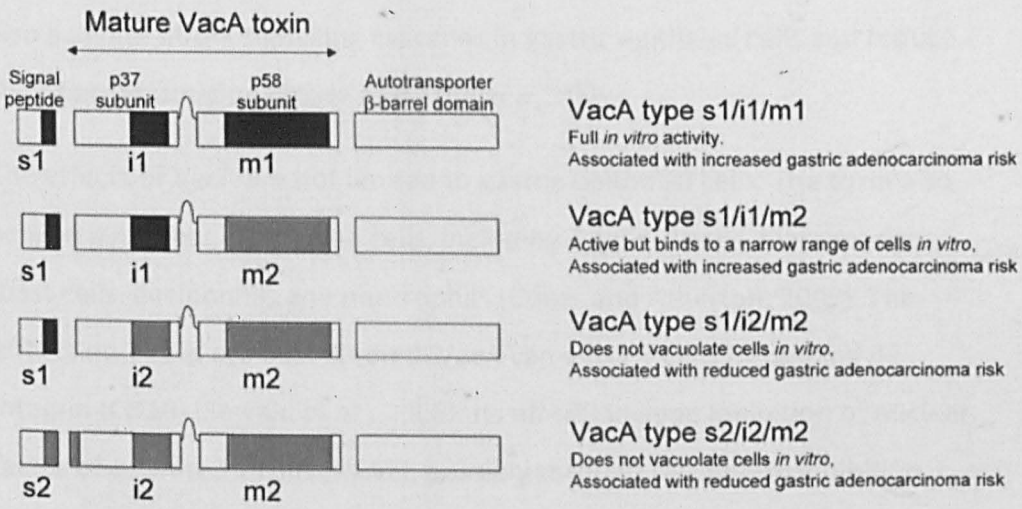


Figure 1.5 Polymorphisms in VacA (from (Robinson and Atherton, 2009)). The main regions of variation are signal (s) region, which determines vacuolating activity, the intermediate (i) region, which determines cytotoxic activity and the midregion (m), which determines the range of cells the toxin is able to bind.

The intermediate region determines cytotoxic activity and strains with i1 alleles are strongly associated with gastric cancer (Rhead et al., 2007). Indeed i-type was found to be an independent risk factor with a stronger influence on gastric carcinoma risk than s-type, m-type or *cag* status (Rhead et al., 2007). The midregion determines the range of cells that the toxin binds, with m1 allele conferring the ability to bind a wider range of cells (Blaser and Atherton, 2004). s1/i1/m1 *vacA* strains are therefore the most pathogenic (Figure 1.5).

VacA is named after its ability to cause vacuolation in gastric epithelial cell lines and classically forms hexameric pores in membranes. These pores allow leakage of cell contents, including urea, which acts as a substrate for urease, allowing *Hp* to buffer itself against the acid gastric environment (Atherton, 2006). VacA localizes to plasma and mitochondrial membranes where it can increase permeability (though pore formation) and activate apoptosis (Cover and Atherton, 2009, Galmiche et al., 2000, Jain et al., 2011). The toxin can also activate MAPK signalling cascades in gastric epithelial cells and reduce their barrier function (Cover and Atherton, 2009).

The effects of VacA are not limited to gastric epithelial cells. The toxin also acts on a number of immune cells, including T cells, B cells, macrophages, mast cells, eosinophils and neutrophils (Cover and Atherton, 2009). The effects on T cells are best studied. VacA can enter T cells by binding β2 integrin (CD18) (Sewald et al., 2008). Its effects include inhibition of nuclear factor of activated T cells (NFAT), possibly through calcineurin inhibition, which modulates IL-2 signalling pathways causing reduced IL-2 secretion and reduced IL-2R expression (Gebert et al., 2003, Cover and Atherton, 2009). This leads to reduced T cell proliferation. VacA can also inhibit antigen processing and presentation (Molinari et al., 1998). Though most of the effects of VacA are immunosuppressive, it can also increase transcription of some pro-inflammatory genes, such as COX-2, and s1m1 strains are more commonly associated with peptic ulcer disease and gastric cancer (Atherton et al., 1995, Blaser and Atherton, 2004). Strains with VacA s1-type are often *cagA*+

(Atherton et al., 1995). It is possible that the immunosuppressive effects of VacA allow persistence of virulent *cagA*+ strains.

1.1.5.3 Duodenal ulcer promoting gene A (DupA)

The duodenal ulcer promoting gene A (*dupA*) virulence factor was only discovered relatively recently (Lu et al., 2005). It has homology to *virB4*, suggesting that, like the *cagPAI*, it may be involved in translocating bacterial products into gastric epithelial cells. However its function has not yet been clarified. The initial study by Lu *et al.* found *dupA*+ strains were associated with increased risk of duodenal ulcer but reduced risk of gastric atrophy and cancer (Lu et al., 2005). It also reported that *dupA*+ strains caused increased IL-8 secretion from gastric epithelial lines, but others have been unable to replicate these findings, though higher *IL8* mRNA level were found in the gastric mucosa of patients infected with *dupA*+ strains compared to those infected with *dupA*- strains (Lu et al., 2005, Hussein et al., 2010). My colleagues found that *dupA*+ strains increased IL-12p40, IL-12p70 and IL-23 secretion by $CD14^+$ mononuclear cells, suggesting that *dupA* may exert its effects on immune cells rather than the gastric epithelium (Hussein et al., 2010)(see Chapter 3).

There has been some controversy about clinical correlations with *dupA* status, as the initial findings have not been replicated in all subsequent study populations. This is likely to be at least in part due to uncertainty about what constitutes a functional *dupA* locus. Hussein *et al.* described a truncated form (*dupA2*) with reduced activity compared to the longer (*dupA1*) form (Hussein et al., 2010). A recent study by Jung *et al.* the presence of *dupA* and 6 adjacent *vir* gene homologues was used as a marker ("complete *dupA* cluster") (Jung et al., 2012). The "complete *dupA* cluster" was associated with increased risk of duodenal ulcer and small increases in IL-8 and IL-12 in the gastric mucosa were also found, though the difference in IL-12 levels was not significant (Jung et al., 2012). Surprisingly this study also found increased gastric atrophy in the *dupA*+ group (Jung et al., 2012). Previous work from the same group found *dupA*+ strains were associated with reduced atrophy in

patients from Japan and Colombia and suggested that *dupA* may help to protect the bacteria from gastric acidity (Lu et al., 2005). This could be beneficial for duodenal ulcer-causing bacteria as this pathology is often associated with antral inflammation and low pH (Figure 1.1).

1.1.5.4 Other virulence factors

Other *Hp* virulence factors include adhesins. *Hp* adhesins can bind to blood group antigens expressed on gastric epithelial cells: blood group antigen binding adhesin (BabA) binds the Lewis b antigen and Lewis x is a ligand for sialic-acid-binding adhesin (SabA). Higher expression of Lewis antigens is associated with increased *Hp* density and active forms of *BabA* are associated with duodenal ulcer and gastric cancer (Atherton, 2006).

Outer inflammatory protein A (OipA) is an outer membrane protein of *Hp*. Active forms are associated with increased mucosal IL-8, neutrophil infiltration and duodenal ulceration (Yamaoka et al., 2000, Atherton, 2006).

A recent meta-analysis found that *induced by contact with epithelium* (*iceA*) polymorphisms had marginal effects on peptic ulcer risk and did not influence gastric cancer risk (Shiota et al., 2012).

Superoxide dismutase and catalase enzymes produced by *Hp* help protect the bacteria against phagocytosis and are also sometimes considered as virulence factors (Dunn et al., 1997). The *Hp*-neutrophil activating protein (HP-NAP) may promote pro-inflammatory $CD4^+IFN\gamma^+$ (Th1) responses in addition to activating neutrophils (Amedei et al., 2006).

1.1.6 Associations of Environmental and Host Factors with *Hp*-related Disease

1.1.6.1 Environmental factors

Smoking is a recognized risk factor for gastric cancer and the risk increases with higher cigarette consumption (Ladeiras-Lopes et al., 2008, Steevens et al., 2010). Smoking had the greatest effect on increasing risk of progression from atrophy to dysplasia, suggesting that it may exert its influence when

progression along the Correa pathway (Figure 1.2) is already underway (Kneller et al., 1992).

Diet can also influence gastric cancer risk with high intake of nitrosamines, salt and red/processed meat increasing risk, whilst fresh fruit and vegetables, vitamin C and "Mediterranean diet" are protective (Liu and Russell, 2008, Buckland et al., 2009, Gonzalez and Riboli, 2010, Peleteiro et al., 2011). A large prospective study: European Prospective Investigation into Cancer and Nutrition (EPIC) has contributed significantly to contemporary knowledge about the influence of diet on gastric cancer risk. Antioxidants reduced risk in smokers, but not non-smokers (Serafini et al., 2012). Though observational studies have shown that antioxidant vitamins are associated with reduced gastric cancer risk, supplementation with vitamin C and E has not protected against cancer in prospective randomized trials (Liu and Russell, 2008, Lin et al., 2009). *Hp* infection leads to reduced vitamin C levels (Rokkas et al., 1995). In addition to its antioxidant acitivity vitamin C can inhibit formation of N-nitroso compounds, inhibit *Hp* growth (at pharmacological doses), enhance the immune response to infection, promote apopotosis and reduce gastric cell proliferation (Zhang and Farthing, 2005).

Nitrosamines may be exogenous, as found in cured and smoked meat and fish, or may be formed by bacterial processing of dietary nitrate (endogenous). Epidemiological studies suggest a link between nitrosamines and gastric cancer but this has not always been borne out in large prospective studies (Keszei et al., 2013, Liu and Russell, 2008, Cross et al., 2010). One prospective study found a small increase in distal gastric cancer risk with high endogenous nitrosamines in those with reduced vitamin C levels or *Hp* infection (Jakszyn et al., 2006), again demonstrating the importance of the combination of infection and other risk factors.

1.1.6.2 Host genetic factors

Host cytokine polymorphisms affect the immune response provoked by *Hp* infection and are known to influence risk of gastric cancer development,

though their effect on peptic ulcer disease is less clear. The best studied examples are polymorphisms in *IL1B,* which encodes IL-1β, and *IL1RN,* the gene encoding the endogenous IL-1 receptor antagonist. IL-1β is a potent suppressor of gastric acid production. Polymorphisms resulting in high IL-1β production increase the risk of hypochlorrydia, atrophy and distal gastric adenocarcinoma (El-Omar et al., 2000). *IL1B* polymorphisms can also influence eradication rates, probably due to effects on gastric pH (Sugimoto et al., 2009). More recently IL-1β has been recognized to promote Th17 differentiation, so it is possible that some of the pro-carcinogenic effects of these polymorphisms could be mediated by Th17 cells (Acosta-Rodriguez et al., 2007a, Serelli-Lee et al., 2012). Polymorphisms in IL-17 itself have also been reported to influence gastric cancer risk (Shibata et al., 2009).

Polymorphisms in the gene for TNF-α, another pro-inflammatory cytokine induced by *Hp* that can suppress acid production, can also increase distal gastric cancer risk (El-Omar et al., 2003). Certain polymorphisms in *IL12A* and *IL12B*, which encode the IL-12p35 and IL-12p40 (also common to IL-23) subunits were also reported to influence distal gastric cancer risk (Navaglia et al., 2005). Polymorphisms in the IL-8 gene are linked with pre-cancerous changes but a positive association with progression to cancer itself has not been found in all populations studied (Lochhead and El-Omar, 2007). Pro-inflammatory polymorphisms in the anti-inflammatory cytokine IL-10 also increase risk of distal gastric cancer (El-Omar et al., 2003).

Polymorphisms in other genes involved in innate immunity can also modulate the risk of developing precancerous changes and cancer. Polymorphisms in the gene encoding Toll-like receptor 4 (TLR4) are associated with gastric cancer (Hold et al., 2007, El-Omar et al., 2008). Genes linked to precancerous changes include the *MPO* gene encoding myeloperoxidase (intestinal metaplasia) and apoptosis-related genes *FAS* and *FASL* (intestinal metaplasia and atrophy) (Hsu et al., 2008a, Hsu et al., 2008b).

Combinations of multiple pro-inflammatory cytokine polymorphisms result in greater increases in gastric cancer risk, e.g. one study calculated an odds ratio of 27 (95% confidence interval 7-100) for combinations of 3 or 4 high-risk genotypes (El-Omar et al., 2003). The combination of virulent infecting *Hp* strain and pro-inflammatory host cytokine polymorphisms resulted in even greater odds of gastric cancer e.g. odds ratio 87 (95% confidence interval 11-679) for *Hp vacA s1*-type and pro-inflammatory *IL1B* gene polymorphism (Figueiredo et al., 2002). Pro-inflammatory cytokine polymorphisms, *Hp* infection and environmental factors can also combine to increase gastric cancer risk, as observed in a recent investigation of the effects of IL-10 gene polymorphisms, *Hp* infection status and smoking on gastric cancer risk (Kim et al., 2012).

$CD4^+$ T helper cells are important sources of many of these cytokines. The T helper cell subsets and their differentiation will be described next.

1.2 $CD4^+$ T HELPER CELL DIFFERENTIATION AND SUBSETS

$CD4^+$ T helper cells have an important role in orchestrating immune responses. They differentiate into different lineages following activation of their T cell receptor (TCR). A number of factors including the local cytokine milieu, the strength of TCR-antigen interaction, the type of antigen presenting cell, strength of co-stimulation and other factors in the local environment determine which type of cell a naive T cell will differentiate into (Kapsenberg, 2003, Shortman and Liu, 2002, Zhu and Paul, 2010). Each $CD4^+$ T cell lineage is characterized by a signature transcription factor and cytokine profile. Mosmann and Coffman proposed a dichotomy of $CD4^+$ T helper cells consisting of IFNγ-secreting Th1 cells and IL-4-secreting Th2 cells which were mutually antagonistic in 1989 (Mosmann and Coffman, 1989). This was the accepted paradigm for over a decade but further $CD4^+$ T cell subsets have now been added to the model.

Regulatory T cells (Tregs) were identified as a $CD4^+CD25^+$ population important for tolerance and protection against autoimmunity (Sakaguchi et

al., 1995). Tregs can either originate directly from the thymus or be induced from differentiated T cells in the periphery. They may be characterized by expression of the transcription factor forkhead box protein 3 (FOXP3) (Hori et al., 2003, Khattri et al., 2003, Fontenot et al., 2003).

Th17 cells were identified as a third effector $CD4^+$ T cell lineage following the discovery of IL-23 in 2000, and are described in detail below (section 1.3) (Oppmann et al., 2000, Aggarwal et al., 2003, Harrington et al., 2005, Park et al., 2005). Their lineage-defining transcription factor is retinoic acid receptor–related orphan receptor (ROR)γt (RORC2 in humans) (Ivanov et al., 2006). TGF-β can differentiate Th2 cells into a proinflammatory IL-9-producing "Th9" population, involved in immunity to intestinal parasites (Dardalhon et al., 2008, Veldhoen et al., 2008b). IL-22 and TNF-α producing clones isolated from psoriatic skin lesions led to the proposal of a "Th22" lineage (Eyerich et al., 2009). Lineage-specific transcription factors have not been identified for either Th9 or Th22.

The most recent $CD4^+$ T cell lineage described are the T follicular helper cells (T_{FH}). The transcription factor B cell CLL Lymphoma-6 (BCL6) has helped establish this as a *bona fide* distinct lineage. T_{FH} cells are found in germinal centres, secrete high levels of IL-21, and provide help to B cells for antibody class-switching and somatic hypermutation (Spolski and Leonard).

A significant challenge for the study of $CD4^+$ T cell subsets is the realization that there is a considerable degree of plasticity between some of the subsets. For example, Th17-derived IFNγ-producers have been widely described, and may express T-bet and lose IL-17-secreting capacity (Lee et al., 2009, Hirota et al., Boniface et al., 2010, Zielinski et al., 2012, Shi et al., 2008, Bending et al., 2009, Annunziato et al., 2007, Cosmi et al., 2011, Nistala et al., 2010) and Tregs can convert to an IL-17-producing phenotype in proinflammatory environments (Xu et al., 2007, Koenen et al., 2008, Voo et al., 2009). Some $CD4^+$ T cell populations are more stable than others. T_{FH} cells appear to be a particularly plastic population (Spolski and Leonard, Nakayamada et al.). On

the other hand Th1 seems to be a relatively stable phenotype (Shi et al., 2008).

The main focus of this thesis is Th17 cells. These will be described in detail in the subsequent sections.

	T helper subset				
	Th1	**Th2**	**Th17**	**T_{FH}**	**iTreg**
Characteristic cytokines	IFNγ	IL-4, IL-5, IL-13	IL-17, IL-17F, IL-22 ..	IL-21	IL-10, TGF-β, IL-35
Key transcription factor	T-bet	GATA-3	RORC2	BCL6	FOXP3
Promotes	Macrophage activation	Antibody production	Neutrophil recruitment and antibacterial peptide expression	B cell help in germinal centres: Class-switching and somatic hypermutation	Inhibits effector Th proliferation and cytokine secretion
Necessary for defence against	Intracellular pathogens	Extracellular parasites	Extracellular bacteria and fungi	Antibody-mediated immunity to viruses and extracellular bacteria	Damage from excess inflammation and cancer immunosurveillance
Result of overactivity	Autoimmunity	Allergy	Autoimmunity	Autoimmunity	

Table 1.1 Key cytokines, transcription factors and activities of the major $CD4^+$ T cell subsets

1.3 TH17 CELLS

1.3.1 Discovery of the Th17 Lineage

IL-23 was discovered in 2000 (Oppmann et al., 2000). It shares the p40 subunit with IL-12 and the IL-12 and IL-23 receptors also have a common subunit (IL-12Rβ1). Some effects previously ascribed to IL-12 and Th1 cells were found to be due to IL-23 and Th17 cells, as earlier experiments blocking p40 or IL-12Rβ1 would have blocked IL-23 as well as IL-12. Experiments using the EAE mouse model of multiple sclerosis found that $p40^{-/-}$ mice were protected from disease but $p35^{-/-}$ mice remained susceptible (Becher et al., 2002, Gran et al., 2002). IL-23 was shown to promote IL-17 producing $CD4^+$ T cells (Aggarwal et al., 2003). Th17 cells are now recognized as a distinct, $CD4^+$ T cell lineage, characterized by the transcription factor RORγt (Harrington et al., 2005, Park et al., 2005, Ivanov et al., 2006).

1.3.2 Th17 Differentiation

Although IL-23 promotes Th17 cells and led to their discovery, IL-23R is not present on naïve T cells and other cytokines are required for the initial differentiation of Th17 cells (Figure 5.1). TGF-β and IL-6 or IL-21 are required for Th17 differentiation in mice (Zhou et al., 2007a, Bettelli et al., 2006, Veldhoen et al., 2006, Nurieva et al., 2007). Pro-inflammatory cytokines, particularly IL-1β, IL-6, and IL-21 favour human Th17 differentiation but the role of TGF-β here has been controversial (Volpe et al., 2008, Acosta-Rodriguez et al., 2007a, Manel et al., 2008, Wilson et al., 2007).

Early studies reported development of human Th17 cells with IL-1β and IL-6 or IL-1β and IL-23 (Acosta-Rodriguez et al., 2007a, Wilson et al., 2007). Manel *et al.* and Volpe *et al.* used serum-free medium and TGF-β blocking antibody to exclude even low concentrations of TGF-β from their T cell differentiation experiments and cord blood to ensure that the T cells were naïve. They reported that TGF-β is required for human Th17 differentiation (Manel et al., 2008, Volpe et al., 2008). Ghoreschi *et al.* demonstrated Th17 differentiation

in the absence of TGF-β in mice, suggesting that maybe the differences in Th17 differentiation between mice and humans are not as great as previously thought (Ghoreschi et al., 2010). In this study more IL-17 producing cells developed when TGF-β was present and IL-23 absent. The cells differentiated in the presence of TGF-β and absence of IL-23 produced IL-10 and were less pathogenic upon adoptive transfer in an EAE model than the Th17 cells differentiated in the absence of TGF-β. Adoptive transfer of the latter led to a higher proportion of IL-17$^+$IFNγ$^+$ cells in the central nervous system (Ghoreschi et al., 2010). Tregs can promote murine Th17 differentiation *in vitro* and *in vivo* which may be at least partly due to their production of TGF-β (Veldhoen et al., 2006, Lohr et al., 2006, Vokaer et al., 2010). Tregs may also favour Th17 differentiation by acting as an IL-2 sink (Chen et al., 2011), as IL-2-deficient conditions promote Th17 cells (Laurence et al., 2007, Lohr et al., 2006). Recent studies indicate that Treg derived TGF-β is not essential for Th17 promotion and Th17 cells themselves are a source of TGF-β which may act in an autocrine manner (Chen et al., 2011, Gutcher et al., 2011). IL-6 in the context of TGF-β or IL-21 can upregulate *IL23R* expression on human CD4$^+$ T cells (Yang et al., 2008a).

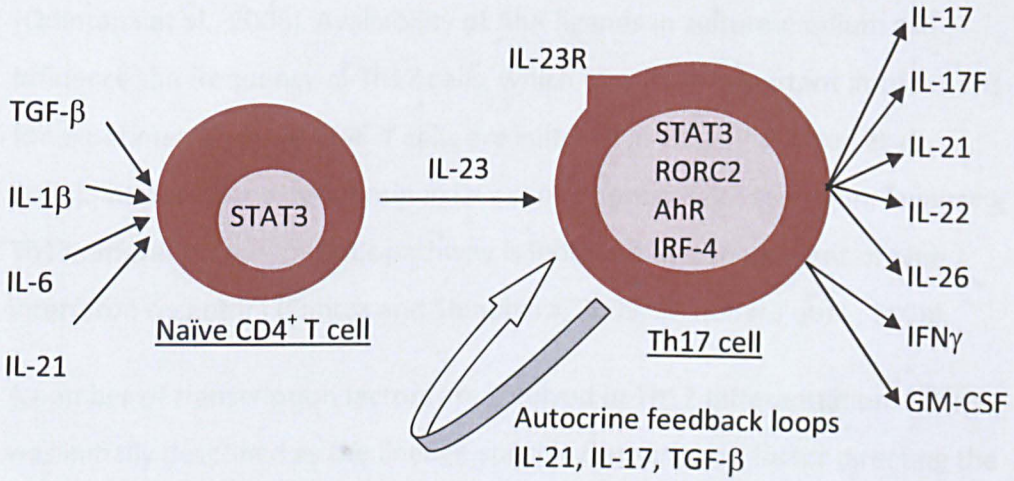


Figure 1.6 Summary of Th17 differentiation and phenotype. IFNγ and GM-CSF are examples of cytokines produced by a more pro-inflammatory subset of Th17 cells promoted by IL-23.

Factors other than cytokines can also influence Th17 differentiation, for example halofuginone can inhibit Th17 differentiation in humans and mice by triggering the amino acid starvation pathway (Sundrud et al., 2009). Digoxin and its synthetic derivatives inhibited Th17 differentiation by antagonizing RORγt activity (Huh et al., 2011). TGF-β is involved in both Th17 and Treg differentiation and the balance of these pro- and anti-inflammatory cells is critical. This is particularly important in the gastrointestinal tract which is populated by millions of commensal bacteria and regularly exposed to foreign antigens in food, but must defend the body against pathogenic infection. During normal homeostasis Treg differentiation is favoured at the expense of Th17 differentiation by retinoic acid, IL-35 and IL-27 (Mucida et al., 2007, Niedbala et al., 2007, Pot et al., 2011). However, if the innate immune system recognizes infected apoptotic cells or Pathogen Associated Molecular Patterns (PAMPs) are recognized by host cell Pattern Recognition Receptors (PRRs), a Th17 response will result (Torchinsky et al., 2009, Nyirenda et al., 2011). Availability of suitable aryl hydrocarbon receptor (AhR) ligands can also

increase Th17 differentiation and was essential for IL-22 secretion in a mouse model (Veldhoen et al., 2008a). Other AhR ligands favour Treg differentiation (Quintana et al., 2008). Availability of AhR ligands in culture medium can influence the frequency of Th17 cells, which may have important implications for experiments where $CD4^+$ T cells are cultured *in vitro* (Veldhoen et al., 2009). Intracellular osteopontin in DCs can suppress IL-27 secretion, favouring Th17 differentiation, but this pathway is inhibited by engagement of type 1 interferon receptors (Cantor and Shinohara, 2009, Shinohara et al., 2008).

A number of transcription factors are involved in Th17 differentiation. RORγt was initially described as the lineage-specific transcription factor directing the Th17 programme (Ivanov et al., 2006). Levels of RORα are also increased in murine Th17 cells, but lack of RORα has little effect on Th17 differentiation, suggesting that it has a minor role in comparison to RORγt (Yang et al., 2008c, Zhou and Littman, 2009). Expression of *RORA*, the human homologue of murine RORα was marginally increased in Th17 and Treg cells compared to Th1 and Th2 cells but levels were much lower and the differences much smaller than found for *RORC2* (Burgler et al., 2009). IL-6, IL-21 and IL-23 all signal via STAT3. These cytokines induce RORγt, which together with STAT3 promote IL-17 expression (Zhou et al., 2007a). The transcription factor IRF-4, which is involved in Th2 differentiation is also required for Th17 differentiation in mice: IRF-$4^{-/-}$ did not develop EAE and had reduced RORγt but increased FOXP3 expression (Brustle et al., 2007). A number of other transcription factors can fine tune Th17 differentiation. The transcription factor c-Maf is increased in murine Th17 cells and c-$Maf^{-/-}$ mice had reduced IL-21 production and reduced numbers of Th17 cells, consistent with the autocrine role of IL-21 in Th17 differentiation (Bauquet et al., 2009). c-Maf has also been implicated in promoting IL-10 production and suppressing IL-22 production in Th17 cells, so it may favour a regulatory Th17 phenotype (Xu et al., 2009, Rutz et al., 2011). The AhR is also expressed in Th17 cells and seems to be particularly important for IL-22 production (Veldhoen et al., 2008a). The

Ets-1 transcription factor negatively regulates Th17 differentiation via effects on the IL-2 pathway (Moisan et al., 2007).

1.3.3 Th17 Phenotype and Plasticity

Th17 cells typically produce IL-17, IL-17F, IL-21 and IL-22 (Acosta-Rodriguez et al., 2007b, Wilson et al., 2007, Volpe et al., 2008), however the exact cytokine profile can vary depending on the conditions under which the cells differentiated and signals the cell receives from its local environment. Other cytokines reported to be produced by Th17 cells include IL-6, TNF-α, CCL20, and IL-26 (Volpe et al., 2008, Langrish et al., 2005, Manel et al., 2008, Wilson et al., 2007). These cytokines are discussed in detail in Chapter 4, with the exception of IL-26. IL-26 is a member of the IL-10 cytokine family. It has no murine homologue and has not been widely investigated but it is increased in inflamed colonic tissue from patients with Crohn's disease and upregulates TNF-α and IL-8 expression in colorectal cancer-derived epithelial cell lines *in vitro* (Dambacher et al., 2009).

GM-CSF production by murine Th17 cells was reported in an early study describing Th17 cells (Langrish et al., 2005). There has been a recent resurgence of interest in GM-CSF as two studies have confirmed its production by murine Th17 cells and shown that it is required for the pathogenic effects of both Th17 and Th1 cells in EAE (El-Behi et al., 2011, Codarri et al., 2011).

Th17 cells producing the regulatory cytokine IL-10 and IFNγ, classically a Th1 cytokine have also been described (Volpe et al., 2008, Peters et al., 2011, Ghoreschi et al., 2010, Zielinski et al., 2012). It is now recognized that not all Th17 cells are the same and some may have more regulatory phenotypes while others are more pro-inflammatory (Peters et al., 2011, Ghoreschi et al., 2010). As described above, Ghoreschi *et al.* found that the presence of TGF-β and absence of IL-23 favoured differentiation of a more regulatory phenotype (Ghoreschi et al., 2010). Zielinski *et al.* reported that IL-1β favoured

differentiation of pro-inflammatory IL-17/IFNγ double producers and inhibited IL-10 production (Zielinski et al., 2012).

The finding of cells that produce classical Treg and Th1 cytokines in addition to IL-17 is not unexpected as it is recognized that there is considerable plasticity in the Th17 lineage. Murine Th17 cells can be converted to IFNγ-producers both *in vitro* and *in vivo* (Shi et al., 2008, Lee et al., 2009, Bending et al., 2009, Hirota et al.). Cells expressing both RORγt and T-bet transcription factors can occur (Zielinski et al., 2012), though T-bet has been shown to inhibit RORγt by sequestering Runx1 (Lazarevic et al., 2011). Boniface *et al.* studied human T cells differentiated under Th17-inducing conditions and identified a T-bet$^+$IFNγ$^+$IL-17$^-$ population with a cytokine profile more similar to Th17 than Th1 cells (Boniface et al., 2010). This suggests that Th17-derived IFNγ-producing T cells may not be classical Th1 cells. There are no reports of Th1 cells converting into Th17 cells (Shi et al., 2008), suggesting that Th1 cells are a more stable population.

Treg cells can convert into IL-17-producing cells in pro-inflammatory environments (Xu et al., 2007, Koenen et al., 2008, Voo et al., 2009). Low frequencies of FOXP3$^+$IL-17$^+$ cells have been identified in human peripheral blood and tonsil tissue (Voo et al., 2009). Despite producing IL-17 these cells have suppressive activity (Voo et al., 2009). A further CD4$^+$ subset, the T follicular helper cells, which secrete high levels of IL-21 and provide B cell help in germinal centres, have been shown to be a highly plastic population that can develop from Th1, Th2 and Th17 cells and vice versa (Lu et al., 2011). Modifications in histone methylation patterns and microRNA expression can effect plasticity by causing epigenetic modification of gene transcription (Wei et al., 2009, Rossi et al., 2011).

1.3.4 Th17 Cells and Host Defense

Th17 cells are important for defense against extracellular bacteria and defense of mucosal surfaces and are also increased in many chronic inflammatory diseases and a number of cancers. Their methods of controlling

infection include recruiting inflammatory cells to the infected site, especially neutrophils, and promoting secretion of anti-bacterial peptides. Studies using cytokine knockout mice have shown that Th17 cytokines are involved in protection against bacterial and fungal infections of the lung and gastrointestinal tract (Ye et al., 2001, Aujla et al., 2008, Ishigame et al., 2009, Conti et al., 2009). IL-$10^{-/-}$ mice infected with *Helicobacter hepaticus* and wild-type mice infected with *Hh* develop IL-23-dependent colitis with Th1, Th17 and $CD4^{+}IL\text{-}17^{+}IFN\gamma^{+}$ mesenteric lymph node cell responses (Kullberg et al., 2006). *Bacteroides fragilis* polysaccharide A can reduce *H. hepaticus*-induced IL-17 production and colitis via $CD4^{+}$ T cell secreted IL-10 (Mazmanian et al., 2008). Murine Th17 responses to pulmonary pathogens, such as *Staphylococcus aureus*, have also been demonstrated (Frank et al., 2012).

Human Th17 recall responses to antigens including *Staphylococcus aureus* and *Candida albicans* have been demonstrated (Zielinski et al., 2012). Patients that have mutations in genes resulting in reduction in Th17 numbers or Th17 function, including IL-17RA, IL-17F, STAT3, Dectin-1 and CARD9 (pattern recognition receptor for *C. albicans* and its signaling molecule) and autoantibodies to IL-17A and IL-17F are prone to chronic mucocutaneous candidiasis with or without *S. aureus* infections (Ma et al., 2008, Milner et al., 2008, Glocker et al., 2009, Ferwerda et al., 2009, Puel et al., 2011). Patients with severe burns are susceptible to *C. albicans* infection and their PBMCs showed a markedly reduced Th17 response to *C. albicans* antigen stimulation *in vitro* (Inatsu et al., 2011). This may be due to inhibition of the Th17 response by IL-10 (Inatsu et al., 2011).

In addition to their classical role in defense against extracellular bacteria and fungi, Th17 responses have also been described in a number of intracellular infections. There is a Th17 response to *Mycobacterium tuberculosis* (TB) and *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) infection, though a significant proportion of the IL-17 response to these mycobacteria is produced by $\gamma\delta$ T cells (Peng et al., 2008, Umemura et al., 2007). $IL\text{-}17^{+}IFN\gamma^{+}$ double cytokine producing $CD4^{+}$ cells were increased in blood and pleural fluid

of patients with active TB and levels were higher in those with severe disease (Jurado et al., 2012). IL-17$^{-/-}$ mice infected with BCG had reduced IFNγ production and impaired granuloma formation compared to their wild-type counterparts (Umemura et al., 2007), demonstrating a role for IL-17 in the pathogenesis of this infection. Human Th17 cells with specificity for HIV-1 and CMV have also been demonstrated (Yue et al., 2008). Th17 cell counts fall at an earlier stage in HIV infection than some other CD4$^+$ T cell subgroups (Prendergast et al., 2010). In addition to direct anti-viral activity Th17 cells may also provide help to CD8$^+$ T cells and B cells (Khader et al., 2009). Th17 cells are also increased in chronic hepatitis B and C infection, where they correlate with liver inflammation and fibrosis, suggesting that with failure to clear the infecting organism the Th17 response may contribute to pathology (Sun et al., 2012, Chang et al., 2012, Ge et al., 2009). Th17 cytokines IL-17 and IL-22 can promote hepatocellular cancer development in this chronic inflammatory context (Gu et al., 2011, Jiang et al., 2011). IL-17 also failed to clear the virus but contributed to pathology in models of murine HSV-1 corneal infection and rhinovirus infection of human primary bronchial epithelial cells (Molesworth-Kenyon et al., 2008, Wiehler and Proud, 2007). Where the host Th17 responses are not able to clear bacterial infection pathology may also result. Examples include chronic biofilm infection in cystic fibrosis and a mouse model of chronic granulomatous disease with defective NADPH oxidase activity (Romani et al., 2008, Dubin et al., 2007).

1.3.5 Th17 Cells and Chronic Inflammatory Disease

Th17 cells have been shown to be increased in a number of chronic inflammatory conditions in humans (Pene et al., 2008). Increased frequencies of Th17 cells are found in intestinal biopsies from patients with Crohn's disease (Annunziato et al., 2007, Kleinschek et al., 2009). A significant proportion of these cells also secrete IFNγ following stimulation with PMA and ionomycin (Annunziato et al., 2007). Polymorphisms in the IL-23R gene and other Th17-related genes including *stat3* and *ccr6* affect risk of Crohn's

disease, further supporting a role for Th17 in its pathogenesis (Duerr et al., 2006, Dubinsky et al., 2007, Barrett et al., 2008).

Patients with multiple sclerosis (MS) had high levels of Th17 cells in their cerebrospinal fluid (CSF), which increased during relapses (Brucklacher-Waldert et al., 2009b). Peripheral blood lymphocytes from relapsing MS patients had increased propensity to develop into $CD4^+IL\text{-}17^+IFN\gamma^+$ double-producers upon expansion with IL-23 *in vitro*. T-bet and RORγt co-staining was also demonstrated in human central nervous system tissue using confocal microscopy (Kebir et al., 2009). IFN-β, a widely used therapy for relapsing-remitting MS, inhibits Th17 differentiation (Ramgolam et al., 2009).

$IL\text{-}17^+$ and $IL\text{-}22^+$ $CD4^+$ T cells are also present at increased levels in the peripheral blood of patients with rheumatoid arthritis and ankylosing spondylitis (Shen et al., 2009). Increased IL-17 concentrations in the synovial fluid of rheumatoid arthritis patients were demonstrated over a decade ago and IL-17 and IL-21 can stimulate osteoclastogenesis (Kotake et al., 1999, Kwok et al., 2012). However, it has been reported that Th1 cells are more abundant than Th17 cells in the joints of patients with rheumatoid arthritis (Yamada et al., 2011). Nistala *et al.* found that a significant proportion of Th17 cells from joints of patients with juvenile idiopathic arthritis (JIA) secreted IFNγ and expressed both Th1 and Th17 transcription factors. A proportion of the $CD4^+IFN\gamma^+IL\text{-}17^-$ cells from the joints of patients with JIA were $CD161^+$ and had higher RORC2, CCR6 and IL-23R expression than the $CD161^-$ Th1 population (Nistala et al., 2010), suggesting that some $CD4^+IFN\gamma^+$ cells in the inflamed joints may be derived from the Th17 lineage. Cosmi *et al.* also reported $CD4^+CD161^+$ "Th1/Th17" and "Th1" cells in the synovial fluid of JIA patients, which correlated with inflammatory markers (Cosmi et al., 2011). This could be consistent with a Th17-derived, $IL\text{-}17^-IFN\gamma^+$ pathogenic cell population.

Th17 cells are also increased in the skin lesions and peripheral blood of patients with psoriasis (Kagami et al., 2010, Zhang et al., 2010a). The Th17

cytokine IL-22 is increased in psoriatic lesions and in the plasma of patients with psoriasis, where it correlated with disease severity and reduced with treatment (Wolk et al., 2006). A $CD4^+IL\text{-}17^-IL\text{-}22^+$ population was identified in the inflamed epidermis of patients with psoriasis and dubbed "Th22" (Eyerich et al., 2009). However no lineage-specific transcription factor has yet been identified for this population. Res *et al.* found that skin-derived Th17 clones could lose IL-17 expression and become IL-22 single-producers (Res et al., 2010), suggesting that "Th22" cells may be a further manifestation of Th17 instability/plasticity.

1.3.6 Th17 and Cancer

Th17 cells have been found to be increased in gastric cancer (Zhang et al., 2008a, Iida et al., 2011) and a number of other human tumours (Ye et al., 2010, Kryczek et al., 2009a, Sfanos et al., 2008, Kryczek et al., 2007). Tumour microenvironments appear to favour recruitment and expansion of Th17 populations (Su et al., 2010). Tumour-infiltrating Th17 cell frequencies and Th17 cell numbers in malignant pleural effusions correlate with survival or slower progression of disease in a number of human cancers and, in mice, Th17 cells can completely eradicate large melanomas (Sfanos et al., 2008, Muranski et al., 2008, Kryczek et al., 2009a, Ye et al., 2010). However, in other malignancies Th17 levels did not affect disease outcome or conversely were associated with more rapid disease progression (Zhang et al., 2010c, Derhovanessian et al., 2009). The role of IL-17 in neoplastic disease is discussed in Chapter 5. A significant proportion of Th17 cells from tumour sites co-express TNF-α, IL-2 or IFNγ (Kryczek et al., 2009a). Studies in ovarian cancer suggest that Th17-derived IL-17 and IFNγ can synergistically increase CXCL9 and CXCL10 expression, which in turn increase $CD8^+$ T cell recruitment (Kryczek et al., 2009a). Th17 cells caused activation of $CD8^+$ T cells with anti-tumour activity in murine lung melanoma (Martin-Orozco et al., 2009). Other Th17 cytokines may also have important roles in cancer. IL-22 appears to have some pro-tumour effects as it can stimulate human pancreatic cancer cells to

produce vascular endothelial growth factor and anti-apoptotic factors (Curd et al., 2012).

1.3.7 Anti-Th17 Treatment in the Clinic

The anti-p40 monoclonal antibody ustekinumab, which inhibits both Th1 and Th17 differentiation has shown promise in randomized trials for Crohn's disease and psoriasis (Sandborn et al., 2008, Krueger et al., 2007, Leonardi et al., 2008, Sandborn et al., 2012). Anti-IL-17 monoclonal antibodies have shown some efficacy in clinical trials for psoriasis, rheumatoid arthritis and uveitis, but were disappointing in Crohn's disease, with a high incidence of infections in the anti-IL-17-treated group (Hueber et al., 2012, Leonardi et al., 2012, Genovese et al., 2010, Hueber et al., 2010). An IL-17R antibody was also effective for the treatment of psoriasis (Papp et al., 2012). IL-21 makes T cells resistant to Treg suppression and may be a further Th17 cytokine therapeutic target in Crohn's disease but this hypothesis has not yet been tested in clinical trials (Monteleone et al., 2009). There is interest in inhibiting transcription factors involved in Th17 differentiation and drugs already in clinical use including simvastatin and digoxin have been shown to do this *in vitro* (Zhang et al., 2008b, Huh et al., 2011). Pioglitazone also inhibited EAE in mice (Klotz et al., 2009). Intravenous immunoglobulin can also interfere with RORC2 and STAT3 expression and Th17 cytokine production, which may account for some of its immunomodulatory effects (Maddur et al., 2011).

1.3.8 Th17 Cell Markers

The study of human Th17 cells has been hampered by the lack of specific cell markers to identify this population. Acosta-Rodriguez *et al.* reported that CCR4 and CCR6 memory $CD4^+$ T cells have a Th17 phenotype (Acosta-Rodriguez et al., 2007b). However, CCR4 and CCR6 can also be expressed by Tregs (Lim et al., 2008). CCR6 has been widely used in conjunction with other markers to help identify Th17 cells (Manel et al., 2008, Boniface et al., 2010, Annunziato et al., 2007).

The IL-23R is upregulated on Th17 cells by IL-6 early in Th17 differentiation (Ivanov et al., 2006, Zhou et al., 2007a). Some investigators have used the IL-23R, at the mRNA level or with polyclonal antibodies for flow cytometry, as a Th17 marker (Annunziato et al., 2007, Wilson et al., 2007). However, no monoclonal anti-IL-23R antibodies were available when this study was designed and the IL-23R is also expressed by other IL-17-secreting cells including $CD8^+$ T cells, $\gamma\delta$ T cells and other innate lymphoid populations, including lymphoid tissue inducer-like cells (Billerbeck et al., 2010, Huber et al., 2009, Sutton et al., 2009, Takatori et al., 2009, Buonocore et al., 2010).

IL-22^+Th17 cells originate from $CD161^+$ precursors and maintain their $CD161^+$ phenotype (Cosmi et al., 2008). CD161 is also expressed on subsets of NK cells and $CD8^+$ T cells and can occur at low levels on Th1 and Th2 clones (Lanier et al., 1994, Cosmi et al., 2008). $CD161^+$ T cells may express IL-17 and/or IFNγ (Cosmi et al., 2008). A significant proportion of $CD161^+$ T cells express the gut homing integrin $\alpha4\beta7$ and high numbers of these cells have been found in inflamed colonic tissue from patients with Crohn's disease (Kleinschek et al., 2009).

Even expression of the Th17 transcription factor RORC2 is not restricted to Th17 cells. RORC2 expression has been described in $CD8^+$ T cells and NKT cells. Its murine orthologue RORγt is also expressed on innate IL-17 producing cells including $\gamma\delta$ T cells and lymphoid tissue inducer-like cells (Burgler et al., 2009, Sutton et al., 2009, Takatori et al., 2009).

Flow cytometry with extracellular CD4 staining and intracellular IL-17 staining may be the most reliable way of identifying $CD4^+$IL-17^+ Th17 cells, but requires the cells to be permeabilized, precluding subsequent functional experiments. As the plasticity and complexity of the Th17 cells is becoming better understood, wider panels of markers are now becoming necessary to further characterize Th17 populations.

1.3.9 Non-Th17 Cell Sources of IL-17

IL-17-secreting $CD8^+$ T cells (Tc17s) develop in similar STAT3-dependent conditions to those that promote Th17 development (Huber et al., 2009). They have low perforin and granzyme expression but usually express IL-23R, CD161, CCR6 and RORC2 and often co-express other inflammatory cytokines such as IFNγ, IL-21, IL-22 and TNF-α (Billerbeck et al., 2010, Kondo et al., 2009, Huber et al., 2009, Ortega et al., 2009). They exhibit some plasticity with the potential to convert to IL-17$^-$IFNγ-producers (Hinrichs et al., 2009, Yen et al., 2009). Tc17 cells are involved in anti-viral immunity (Billerbeck et al., 2010, Intlekofer et al., 2008, Grafmueller et al., 2012, Hamada et al., 2009). They have also been detected in a number of tumours and can mediate anti-tumour immunity in mouse models (Kuang et al., 2010, Hinrichs et al., 2009, Garcia-Hernandez et al., 2010). Tc17s are implicated in colitis and EAE mouse models (Tajima et al., 2008, Huber et al., 2009) and are increased in the skin of patients with psoriasis and in the blood of patients with relapsing multiple sclerosis and neuromyelitis optica (Ortega et al., 2009, Res et al., 2010, Wang et al., 2011).

T cells expressing the γδ T cell receptor (TCR) are involved in innate immune responses. They can also express the IL-23R, RORγt, CCR6 and AhR and produce IL-17, IL-21 and IL-22 (Sutton et al., 2009, Martin et al., 2009). These cells express TLR1, TLR2 and dectin-1, allowing them to interact directly with some pathogens (Lochner et al., 2008, Martin et al., 2009). They contribute significantly to IL-17 secretion in murine bacterial infection and are expanded in the blood of patients with certain bacterial and viral infections (Lockhart et al., 2006, Caccamo et al., 2011, Fenoglio et al., 2009, Shibata et al., 2007). IL-17^+ γδ T cells appear to contribute to pathology in mouse models of autoimmune disease, including EAE, collagen induced arthritis and colitis (Sutton et al., 2009, Do et al., 2011, Ito et al., 2009). They are increased in the peripheral blood of patients with ankylosing spondylitis but their role in

human disease has not yet been fully elucidated (Kenna et al., 2012, Ito et al., 2009).

Invariant natural killer T (iNKT) cells are a further innate $CD3^+$ source of IL-17. They recognize glycolipid antigens, such as α-galactosylceramide, presented in the context of CD1d. A subset of iNKT cells in murine lymphoid tissue can express the IL-23R, RORγt, CCR6 and CD103 and rapidly produce IL-17 in response to α-galactosylceramide or TCR or IL-23R ligation (Rachitskaya et al., 2008, Doisne et al., 2009). However, NKT cells are reported to account for <2% T cells in the gastric mucosa of patients without *Hp* infection, with no significant increase in gastric biopsies from *Hp*-infected patients (O'Keeffe et al., 2008).

A number of non-T cell sources of IL-17 have also been described and recently it has been proposed that these IL-17- and/or IL-22-producing innate lymphoid cells (ILCs) should be termed group 3 ILCs, or ILC3s (Spits et al., 2013). This classification also encompasses ILC1s, including NK cells, that are defined by their production of IFNγ, and ILC2s, which produce Th2-associated cytokines (Walker et al., 2013, Spits et al., 2013). An increased proportion of ILC2s was found in nasal polyps from patients with chronic rhinosinusitis (Mjosberg et al., 2011).

The ILC3 subset expresses RORγt and also depends on IL-7Rα (CD127) for its development. It may be further subdivided into lymphoid tissue-inducer (LTi) cells, natural cytotoxicity triggering receptor $(NCR)^+$ and NCR^- populations (Walker et al., 2013, Spits et al., 2013). LTi cells are required for the development of secondary lymphoid tissue (Eberl et al., 2004). Some murine LTi cells express CD4, but human LTi cells are $CD4^-$ (Cupedo et al., 2009). Takatori *et al.* were the first to report an $IL\text{-}23R^+CCR6^+AhR^+$ lymphoid tissue inducer-like (LTi-like) population (NCR^+ ILC3s, also previously referred to as NK22 cells) that produced IL-17 and IL-22 in response to zymosan stimulation in mice (Takatori et al., 2009). A similar population has been identified in human fetal tissue and is thought to be the precursor to $NKp46^+$ cells (NCR^+

ILC3s) which produce IL-17 and IL-22 in humans, but IL-22 only in mice (Cupedo et al., 2009, Satoh-Takayama et al., 2008). Buonocore *et al.* described yet another IL-23-responsive $THY1^{+}SCA1^{+}CD3^{-}CD4^{-}KIT^{-}$ innate IL-17-producing population in the intestine of mice with infection-driven colitis (NCR^{-} ILC3s), which also produces IFNγ (Buonocore et al., 2010). Depletion of this population ameliorated colitis in *H. hepaticus* $Rag2^{-/-}$ mice (Buonocore et al., 2010). An equivalent NCR^{-} ILC3 population has not yet been described in humans.

The relationship between these populations and their plasticity is not yet fully understood, but interestingly, it seems that ILC3 cells may have the potential to convert into ILC1s (Walker et al., 2013). IL-7 and intestinal microbiota appear to stabilize the $ROR\gamma t^{+}$ ILC3 phenotype (Vonarbourg et al., 2010, Cella et al., 2010). Vonarbourg *et al.* fate-mapped LTi-like $NCR^{-}ROR\gamma t^{+}$ ILC3s from the lamina propria of the small intestine of $Rorc^{gfp/+}$ mice and found that they could develop into IL-22-producing NCR^{+} ILC3s. A proportion of these subsequently lost RORγt expression and acquired IFNγ-secreting capacity, a phenomenon that was accelerated by IL-12 and IL-15 (Vonarbourg et al., 2010). Cella *et al.* used NCR^{+} ILC3s isolated from human tonsils to demonstrate similar plasticity of human ILC3s. In this study deviation towards an IFNγ-producing phenotype was favoured by IL-2 or IL-23 (Cella et al., 2010).

Additionally paneth cells in the murine intestine may act as a source of IL-17 (Takahashi et al., 2008). Neutrophils may also produce IL-17 under some circumstances (Werner et al., 2011).

1.4 THE IMMUNE RESPONSE TO HELICOBACTER PYLORI

1.4.1 The Innate Immune Response to *Helicobacter pylori*

The innate immune response is the first line of defence. It can respond immediately but is not pathogen-specific and has no memory. Innate immune responses can help prime adaptive immune responses. This section will discuss how the innate immune system recognizes pathogens, and its effector proteins and cells.

1.4.1.1 The mucus layer and epithelial barrier

The acid environment of the stomach forms a chemical barrier to infection with most organisms and the mucus layer and epithelium form additional physical barriers. Neither of these structures are inert barriers. Most *Hp* is found in the mucus layer which is made up of mucins (heavily glycosylated proteins). This is a dynamic structure that can transport trapped debris. The main mucins expressed in adult gastric mucosa are MUC5AC and MUC6 which are secreted by the surface epithelium and glands respectively (De Bolos et al., 1995, Lindén et al., 2010). Mucin expression may be altered in precancerous conditions and gastric cancer (Lindén et al., 2010). Mucins can bind *Hp*, preventing it reaching the epithelium (Lindén et al., 2010). Gastric mucins express Lewis antigens. Lewis^b binds the *Hp* virulence factor BabA (see section 1.1.5.4). Gastric tissue lacking Lewis^b expression did not bind *Hp in vitro* and a study of children with *Hp* infection found higher *Hp* density in Lewis^b-negative children (Lindén et al., 2010, Boren et al., 1993). The mucous layer acts as a matrix for antimicrobial peptides and MUC6 itself has antimicrobial activity (Kawakubo et al., 2004). Patients with Sjogren's syndrome, who have reduced mucous secretion, have increased prevalence and titres of anti-*Hp* antibodies, consistent with a protective role for the mucus (El Miedany et al., 2005).

The gastric epithelium consists of a sheet of polarized cells bound together by tight junctions. The *Hp* virulence factor CagA is able to associate with the tight-junction scaffolding protein ZO-1 to disrupt the apical-junctional complex allowing access to the gastric mucosa (Amieva et al., 2003). Gastric epithelial cells secrete a number of cytokines including IL-1β, IL-6 and IL-8 (Lindholm et al., 1998). They are also a key site for recognition of invading pathogens and express a number of pattern recognition receptors (PRRs) as described below.

1.4.1.2 Pattern Recognition Receptors

PRRs such as toll-like receptors (TLRs) and intracellular nucleotide oligomerization domain receptors (NODs) recognize conserved pathogen

associated molecular patterns (PAMPs), such as lipopolysaccharide, flagellin, muramyl dipeptide and CpG DNA motifs, not found in the human host (reviewed in (Kawai and Akira, 2010)). This allows the innate immune system to recognize invading pathogens as foreign early in infection. Recognition of PAMPs on apoptotic cells can trigger the combination of cytokines required for Th17 differentiation (Torchinsky et al., 2009).

The ligands for PRRs expressed by *Hp* are less potent that those expressed by many other bacteria, which may aid immune evasion, but are nonetheless important for triggering of the innate immune response (Gewirtz et al., 2004, Lee and Josenhans, 2005). Gastric epithelium expresses TLR 4, 5 and 9 and immune cells that may be present in the gastric mucosa also express combinations of TLRs (Schmausser et al., 2004, Kadowaki et al., 2001, O'Mahony et al., 2008). A subgroup of γδ T cells express TLR1, TLR2 and dectin-1 (which binds β-glucan), and can produce IL-17 upon ligation of these receptors, without the need for T cell receptor binding (Martin et al., 2009). MyD88 signalling pathways are triggered by all the TLRs with the exception of TLR3. DCs from MyD88$^{-/-}$ mice had impaired upregulation of activation markers and reduced cytokine secretion in response to *Hp* (Rad et al., 2007). Using DCs from further knockout mice, the same group found that TLR2 was the major surface PRR for *Hp*, with TLR4 making a minor contribution, though TLR4 polymorphisms have been linked to gastric cancer and its precursors (El-Omar et al., 2008, Rad et al., 2009, Hold et al., 2007). Intracellular TLR9 also contributed by binding *Hp* DNA (Rad et al., 2009). TLR2 can trigger anti-inflammatory responses in mice but activation of human TLR2 seems to have more pro-inflammatory effects (Rad et al., 2009, Wang et al., 2010, Zhang et al., 2010b, Nyirenda et al., 2011, Amedei et al., 2006, Sayi et al., 2011).

In addition to TLR9, as discussed above, NOD1 is an important intracellular PRR for recognition of peptidoglycan delivered to gastric epithelial cells via the type IV secretion system encoded by the *cag*PAI (Figure 1.4). NOD1 binding triggers NF-κB activated proinflammatory pathways. Van Beelen *et al.* found that a range of bacteria could stimulate IL-17 production in memory T

cells via NOD2 stimulation, particularly Gram negative bacteria, but *Hp* was not included in their panel (van Beelen et al., 2007).

1.4.1.3 Antimicrobial peptides

The antimicrobial peptides are cationic and can disrupt bacterial cell membranes (Zasloff, 2002). Some antimicrobial peptides also have chemotactic activity, for example β-defensin 2 (hβD2) is a ligand for CCR6 (Yang et al., 1999).

IL-17 and IL-22 can upregulate expression of a number of types of peptide with direct antibacterial activity, including defensins, calgranins and mucins (Gaffen, 2008, Liang et al., 2006). The β-defensins are probably the best studied antimicrobial peptides in the context of *Hp* infection, particularly hβD2. Expression of hβD2 is inducible, and upregulated by *Hp,* whereas expression of hβD1 is constitutive (Bauer et al., 2013). *In vitro* studies suggest that *cag*PAI mediated activation of NOD1 is required for hβD2 expression (Boughan et al., 2006, Grubman et al., 2010). IL-17 can upregulate hβD2 via JAK and NF-kB pathways (Kao et al., 2004). Upregulation of hβD4 in gastritis, including *Hp*-related gastritis, has also been reported, with reduction in levels following eradication (Otte et al., 2009). Conversely, hβD3 expression appears to be reduced in chronic *Hp* infection via a *cag*PAI-mediated mechanism (Bauer et al., 2012, Bauer et al., 2013). MyD88$^{-/-}$ mice had reduced levels of the antibacterial peptide lipocalin 2 and increased *H. felis* colonization density in addition to reduced IL-17 and IL-22 levels, indicating that TLR signalling is linked to expression of some antibacterial peptides (Obonyo et al., 2011).

Other antimicrobial peptides studied in *Hp* infection include the neutrophil-derived α-defensins 1, 2 and 3, which were increased in the gastric juice of *Hp*-infected patients and fell after eradication, and the cathelicidin LL-37 (also known as human cationic antimicrobial peptide 18), which was increased in the epithelium and gastric secretions of *Hp*-infected patients (Isomoto et al., 2004, Hase et al., 2003).

1.4.1.4 Cells of the innate immune system

Hp infection leads to infiltration of lymphocytes, granulocytes and monocytes (Robinson et al., 2007, Kusugami et al., 1999, Itoh et al., 1999). Innate lymphocytes include NK cells, NKT cells, γδ T cells and lymphoid tissue inducer-like (LTi-like) cells. Relatively little is known about these cells in the context of *Hp* infection. NK cell numbers in the gastric mucosa are low and studies have given mixed results regarding whether or not they increase in *Hp* infection (Itoh et al., 1999, Fan et al., 1994, Agnihotri et al., 1998).

O'Keefe *et al.* found low numbers of NKT cells in the gastric mucosa with no increase in *Hp* infection (O'Keeffe et al., 2008). Futagami *et al.* found increased γδ T cells only in patients with severe gastritis or lymphoid follicles (Futagami et al., 2006). However, Hatz *et al.* found no increase in γδ T cells in the lamina propria and Trejdosiewicz *et al.* found no increase in γδ T cells in the gastric epithelium in *Hp* infection (Hatz et al., 1996, Trejdosiewicz et al., 1991).

Neutrophils and macrophages infiltrate the stomach within 2 days of *Hp* infection of mice (Algood et al., 2007). Neutrophils may mediate protective responses in mouse vaccination models (DeLyria et al., 2009). IL-8 is an important neutrophil chemokine and IL-17 upregulates genes involved in neutrophil recruitment and activation (Gaffen, 2008). Neutrophil infiltration is graded for the "activity" component of the updated Sydney score (Dixon et al., 1996).

Like neutrophils, macrophages are phagocytes. Phagocytosis and death due to reactive oxygen species (ROS) within the resultant phagosomes is an important bacterial killing mechanism, but *Hp* tries to evade this using its catalase activity (Ramarao et al., 2000).

Macrophages can be divided into M1 macrophages, which are proinflammatory and microbicidal, and M2 macrophages which are involved in resolution of inflammation and secrete IL-10. Markers of both M1 and M2 macrophages are increased in the gastric mucosa of *Hp*-infected patients, but

levels of inducible nitric oxide synthase, which is associated with M1 macrophages, were particularly high in *Hp*-infected patients with precancerous changes (Quiding-Jarbrink et al., 2010). The macrophage-derived chemokine MIP-1α is increased in *Hp*-infection (Kusugami et al., 1999, Yamaoka et al., 1998). Macrophages can also act as antigen-presenting cells.

Dendritic cells, often described as professional antigen presenting cells, are specialized to take up and process antigen, then present it to T cells in the context of MHC. They are known to infiltrate the *Hp*-infected gastric mucosa and can take up *Hp* and its virulence products (Necchi et al., 2009, Bimczok et al., 2010, Khamri et al., 2010). These cells and their responses to *Hp* are discussed in detail in Chapter 3.

Mast cells are also increased in *Hp*-infected gastric mucosa (Bamba et al., 2002). They are able to recognize pathogens directly by receptors including TLR2 and TLR4 and have pleiotropic effects on immune and other cells involved in mucosal immunity (Abraham and St. John, 2010). Their role in *Hp* infection is not entirely clear but they were found to be essential for clearance of *Hp* in a mouse vaccination model (Velin et al., 2005).

1.4.2 The Adaptive Response to *Helicobacter pylori*

The adaptive immune response consists of $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cell and B cell responses. B cells can develop into antibody-producing plasma cells. Specificity and memory are key features of the adaptive immune response. Clonal expansion allows rapid generation of a pool of lymphocytes specific for a pathogen to which the host has previously been exposed. Studies in mice indicate that Peyer's patches (PP) in the small intestine are an important site for induction of the adaptive immune response to *Helicobacter* species, with reduced gastritis, lymphocyte infiltration and antibody responses in PP-null mice (Nagai et al., 2007, Kiriya et al., 2007), but it is not known if this is also the case for human *Hp* infection.

1.4.2.1 Humoral immunity

Mucosal and systemic IgG and IgA (particularly important for mucosal immunity) responses are mounted to *Hp*. There is some evidence that anti-*Hp* IgA antibodies in maternal milk can delay *Hp* colonisation of breastfed babies (Thomas et al., 1998). However, B cell knockout mice were protected against *Hp* challenge as well as wild-type mice in a vaccination model (Ermak et al., 1998). Selective IgA deficiency is relatively common, but rates of *Hp* infection are not increased in these patients, despite a lack of compensatory increase in IgG (Bogstedt et al., 1996). Levels of IgA specific for certain *Hp* antigens were found to correlate with grade of gastritis, suggesting that they act as a marker of infection rather than contributing to protective immunity (Hayashi et al., 1998). There is also interest in identifying antibody responses to components of *Hp* that may act as serological markers to help stratify for gastric cancer risk, perhaps as part of a multiplex panel (Gao et al., 2009, Anderson et al., 2006). Sayi *et al.* reported that B cells stimulated by *H. felis* TLR2 ligands induced an IL-10-secreting Tr1 population that could suppress the development of preneoplastic pathology (Sayi et al., 2011). This suggests that B cells may have other immunoregulatory roles separate to their antibody production but it is not yet clear whether this is also the case in human *Hp* infection.

Serology is often used in clinical practice as a screening test for *Hp*. *Hp*-specific antibodies are still present in a significant proportion of patients up to 24 months after successful eradication, so other tests are preferable if confirmation of successful eradication is required (Kato et al., 1999).

1.4.2.2 CD4⁺ T cell response to Hp

T cells account for the largest component of the inflammatory cell infiltrate in *Hp*-associated gastritis (Itoh et al., 1999). Studies with MHC II deficient mice found $CD4^+$ T cell responses to be crucial for protection in mouse vaccination models (Ermak et al., 1998, Pappo et al., 1999), with density of gastric T cells correlating with degree of protection (Ermak et al., 1998). $CD4^+$ T cells are

required for development of gastric pathology in mouse *Hp* and *H. felis* infection models (Eaton et al., 2001, Roth et al., 1999). Nagai *et al.* found that transfer of naïve $CD4^+$ T cells into $Rag2^{-/-}$ mice two months after *Hp* infection induced gastritis (Nagai et al., 2007). A recent study by Hitzler *et al.* confirmed that α/β T cells are required to reduce *H. felis* colonization and for development of *H. felis*-induced precancerous changes (Hitzler et al., 2012a). The type of Th response appears to have a large impact on disease development and severity of gastritis.

1.4.2.3 Th1 response

Strong evidence has now accumulated for both mucosal and peripheral blood $CD4^+IFN\gamma^+$ (Th1) responses to *Hp* (D'Elios et al., 1997, Pellicano et al., 2007, Robinson et al., 2008, Ren et al., 2000, Quiding-Jarbrink et al., 2001b, Kenefeck, 2008), though data from early experiments were not conclusive. Early studies isolated cells from the gastric mucosa and stimulated with PMA/ionomycin (Sommer et al., 1998, D'Elios et al., 1997, Bamford et al., 1998). However, over 70% $CD4^+$ T cells from uninfected patients also secreted IFNγ, suggesting that gastric T cells may be intrinsically Th1 polarized or PMA/ionomycin may disproportionately stimulate an IFNγ response (Itoh et al., 1999). ELISPOT indicated that there were more IFNγ secreting cells in *Hp*-negative gastritis than *Hp*+ gastritis (Karttunen et al., 1995) and lamina propria cells from uninfected patients secreted more IFNγ than those from *Hp*+ patients, upon culture for 3 days with *Hp* stimulation, as measured by ELISA (Fan et al., 1994). This could be due to suppression of the IFNγ response by other factors induced by *Hp* e.g. Tregs/anti-inflammatory cytokines and suggests that an IFNγ response may occur in gastric inflammation due to other causes.

Antigen stimulation may be more physiological and less likely to skew the T helper cell distribution. Over 80% of $CD4^+$ cells cloned from the gastric mucosa produced IFNγ upon stimulation with *Hp* antigen (D'Elios et al., 1997). My colleagues found an increase in $CD4^+IFN\gamma^+$ lymphocyte frequencies with

antigenic stimulation in *Hp*+ but not uninfected patients (Robinson et al., 2008). Western blotting experiments indicate STAT4 (a signaling molecule involved in IFNγ production) and T-bet (Th1 transcription factor) are increased in the *Hp*-infected gastric mucosa compared to uninfected tissue, with uninfected patients with gastritis having intermediate levels (Pellicano et al., 2007). Immunohistochemistry has also demonstrated upregulation of IFNγ in the *Hp*-infected gastric mucosa (Lindholm et al., 1998, Holck et al., 2003).

IFNγ levels correlate with inflammation and pathology in humans (Lindholm et al., 1998, Lehmann et al., 2002, Holck et al., 2003, Pellicano et al., 2007) and animals (Yamaoka et al., 2005, Sayi et al., 2009) infected with *Helicobacter* species. Studies using IL-12$^{-/-}$ and IFNγ$^{-/-}$ indicate that protection against *Hp* in mouse vaccination models is Th1-dependent (Akhiani et al., 2002, Shi et al., 2005). IFNγ levels were higher in Mongolian gerbils with ulcers (Yamaoka et al., 2005). T-bet$^{-/-}$ and IFNγ$^{-/-}$ mice infected with *H. felis* were protected against the development of precancerous changes and cancer compared to wild-type mice (Stoicov et al., 2009, Sayi et al., 2009). However SCID mice did develop gastritis upon transfer of T-bet$^{-/-}$ T cells, suggesting that Th1-independent mechanisms can contribute to *Hp*-induced gastric inflammation (Eaton et al., 2006). Hitzler *et al.* found no difference in *Helicobacter* colonization densities between wild-type, p19$^{-/-}$ and p35$^{-/-}$ mice, suggesting that factors other than IL-23 and IL-12 can differentiate/expand Th17 and Th1 cell populations, or other lineages are involved in control of *Helicobacter* infections (Hitzler et al., 2012a). Macrophage inhibitory factor (MIF) knockout mice had reduced inflammation, IFNγ and T-bet expression, suggesting that MIF is a key factor in the Th1 response in this model (Wong et al., 2009). The *Hp* neutrophil activating protein (NAP) promotes Th1 responses (Amedei et al., 2006). On the other hand cyclooxygenase-2, which is increased in the *Hp*-infected gastric mucosa, has an inhibitory effect on Th1 differentiation (Pellicanò et al., 2007, Meyer et al., 2003).

1.4.2.4 Th17 response

IL-17/Th17 responses have been linked to protection in mouse vaccination models (DeLyria et al., 2009, Velin et al., 2009). IL-17 is increased in the human *Hp*-infected gastric mucosa (Luzza et al., 2000, Serelli-Lee et al., 2012). In a small study Caruso *et al.* reported increased Th17 cells in *Hp*-infected gastric mucosa but Serelli-Lee *et al.* reported that though Th17 cells were increased in those with previous *Hp* infection, there was no significant increase in Th17 levels in patients with active *Hp* infection compared to those that had never been infected (Serelli-Lee et al., 2012). The current literature on Th17 cells in *Hp* infection is described in detail in Chapter 6.

1.4.2.5 Regulatory T cell response

Raghavan *et al.* demonstrated that athymic mice reconstituted with lymph node cells depleted of $CD25^+$ T cells had lower density of *Hp* colonization and more severe gastritis than those reconstituted with undepleted lymph node cells (Raghavan et al., 2003). A number of subsequent studies have shown increased numbers of $FOXP3^+$ Tregs in the *Hp*-infected gastric mucosa in both mice and humans (Robinson et al., 2008, Lundgren et al., 2005, Kandulski et al., 2008, Hussain, 2012, Jang, 2010, Rad et al., 2006). In keeping with Raghavan *et al's.* findings depletion of Tregs in mice led to severe gastritis and reduced *Hp* colonization densities (Rad et al., 2006, Kao et al., 2010). Tregs have also been reported to be associated with higher *Hp* colonization densities in humans (Kandulski et al., 2008).

Studies in mice indicate that *Hp*-stimulated DCs favour Tregs differentiation as adoptive transfer of *Hp*-stimulated DCs reduced the IL-17/Foxp3 ratio (Kao et al., 2010). However Mitchell *et al.* report that though *Hp*-stimulated DCs caused proliferation of Tregs, they also reduced their suppressive function, an effect that was IL-1β-mediated (Mitchell et al., 2012).

The influence of *Hp* on DCs and T cell differentiation is discussed in detail in Chapter 3. In addition to cytokines, tissue factors such as retinoic acid favour

Treg differentiation (Mucida et al., 2007). Reduced retinoic acid is associated with increased inflammation, precancerous changes and cancer (Matsumoto et al., 2005a), which may be partly due to its pro-Treg properties. B7-H1 (ligand for programmed death 1 receptor on T cells) expression on gastric epithelial cells following exposure to *Hp* promotes Tregs (Beswick et al., 2007).

In addition to $Foxp3^+$ Tregs, IL-10-secreting $CD4^+CD25^+Foxp3^-$ cells, consistent with a Tr1 Treg phenotype have been reported in the *H. felis* mouse model (Sayi et al., 2011). IL-10-secreting Tregs are also present in the human *Hp*-infected gastric mucosa (Robinson et al., 2008, Hussain, 2012). IL-10 appears to be an important anti-inflammatory mediator as $IL\text{-}10^{-/-}$ mice had more severe gastritis (Matsumoto et al., 2005b, Chen et al., 2001). This resulted in reduced *Hp* colonization densities, with complete eradication of the infection in some models (Matsumoto et al., 2005b, Chen et al., 2001). The anti-inflammatory mediator TGF-β is also increased in the *Hp*-infected gastric mucosa and correlated with FOXP3 expression (Li and Li, 2006, Robinson et al., 2008, Kandulski et al., 2008).

The cell surface molecule CTLA-4 is also expressed on a proportion of Tregs in the human *Hp*-infected gastric mucosa (Hussain, 2012). Mice injected with anti-CTLA-4 prior to *Hp* challenge responded with increased inflammation and reduced bacterial loads, indicating that CTLA-4 is a further mechanism by which Tregs can exert suppressive effects in the context of *Hp* infection (Anderson et al., 2006).

Generation of adenosine catalyzed by CD39 and CD73 expressed on $Foxp3^+$ Tregs has been described as another immunosuppressive mechanism (Deaglio et al., 2007). $CD73^{-/-}$ mice infected with *H. felis* had increased inflammation and reduced bacterial colonization, indicating that they had reduced anti-inflammatory responses compared to the wild-type control mice (Alam et al., 2009). Fletcher *et al.* demonstrated that $CD39^+FOXP3^+$ Tregs can suppress IL-17 production by T effector cells (Fletcher et al., 2009).

Helios is Treg marker that was initially thought to differentiate between thymus-derived and peripherally induced Tregs (Thornton et al., 2010). It is found on Tregs in the *Hp*-infected gastric mucosa but has now shown to be induced in activated and proliferating T cells bringing into question its specificity for thymus-derived "natural" Tregs (Hussain, 2012, Akimova et al., 2011).

Children mount stronger Treg responses to *Hp* infection than adults and it is thought that this may explain their lower incidence of *Hp*-related gastric pathology (Harris et al., 2008, Freire de Melo et al.). High gastric Treg levels are associated with reduced risk of peptic ulcer disease (Robinson et al., 2008). The role of Tregs in *Hp*-induced disease is discussed in detail in Chapter 6, section 6.1.2.

Increased levels of IL-10-secreting FOXP3$^-$ Tregs, consistent with a Tr1 phenotype, have also been found in the peripheral blood of *Hp*-infected patients (Kenefeck, 2008, Greenaway et al., 2011, Satoh et al.). The systemic increase in Tregs during *Hp* infection could explain the increased risk of allergic disease found in the absence of *Hp* infection (Oertli et al., 2012, Blaser et al., 2008, Amberbir et al., 2011). The peripheral blood Treg response to *Hp* infection is discussed in detail in Chapter 4.

1.4.2.6 Th2 response

Studies of unstimulated gastric cells and studies using PMA/ionomycin mitogenic stimulation found little or no IL-4/Th2 response to *Hp* infection (Sommer et al., 1998, Bamford et al., 1998, Itoh et al., 1999, Lindholm et al., 1998, Luzza et al., 2001, Shimizu et al., 2004). However an IL-4/Th2 response was observed when gastric cells from *Hp*-infected patients were stimulated with *Hp* antigen *in vitro* (Robinson et al., 2008, Marotti et al., 2008). IL-4$^{-/-}$ mice had more severe gastritis and higher levels of IFNγ than wild-type mice (Smythies et al., 2000), suggesting that IL-4 might have a protective role. *Hp* can induce the gastric epithelium to produce thymic stromal lymphopoietin, which promotes Th2 responses (Kido et al., 2010). Phase-variable expression

of lipopolysaccharide Lewis antigens in a single strain of *Hp* can alter the balance of the host Th1/Th2 response induced (Bergman et al., 2004). IL-4 findings in *Hp*-infected gastric mucosa are discussed further in section 5.1.3.3.

1.4.2.7 CD8+ T cell response

$CD8^+$ T cells recognize antigen-derived peptide presented on MHC class I and kill the antigen-bearing cells using granzyme and perforin. They are therefore suited to defense against intracellular pathogens, such as viruses. Most $CD8^+$ T cells secrete IFNγ, TNF-α and lymphotoxin, however some $CD8^+$ T cells have different cytokine profiles and may secrete cytokines including IL-4 or IL-17 (Mosmann et al., 1997, Huber et al., 2009).

Increased $CD8^+$ T cells have also been reported in the *Hp*-infected gastric mucosa (Nurgalieva et al., 2005, Hatz et al., 1996, Bamford et al., 1998). In a small study (8 *Hp*+ and 8 uninfected patients) Fan *et al.* reported that lamina propria $CD8^+$ T cells were increased in the *Hp*-infected gastric mucosa but $CD4^+$ T cells were not (Fan et al., 1994). Others have found that the CD4:CD8 T cell ratio is increased in *Hp* infection (Bamford et al., 1998, Wu et al., 2007). Bamford *et al.* found that $CD8^+$ T cells accounted for a higher proportion of the gastric T cells than $CD4^+$ T cells and formed a substantial proportion of the IFNγ-secreting cells (Bamford et al., 1998). Lundin *et al.* found reduced numbers of activated $CD8^+$ T cells and increased IL-10 levels in the gastric mucosa of patients with gastric cancer, which could be consistent with *Hp*-induced IL-10 suppressing anti-tumour immunity (Lundin et al., 2007).

1.5 HYPOTHESES AND AIMS

1.5.1 Hypotheses

The main hypotheses to be explored are as follows:

- Dendritic cells produce IL-23 upon exposure to *Hp*, supporting Th17 differentiation and expansion.
- A Th17 response is present in the peripheral blood of *Hp*-infected patients.
- IL-17/Th17 cells are an important component of the proinflammatory response to human *Hp* infection and lead to recruitment of proinflammatory cells, including neutrophils and $CCR6^+$ lymphocytes to the *Hp*-infected stomach.
- Th17-associated inflammation leads to increased risk of peptic ulcer disease and Th17 responses may also have a role in carcinogenesis in a subgroup of *Hp*-infected patients.

1.5.2 Aims

- To investigate IL-23 production by DCs in response to *Hp,* and IL-23 levels relative to IL-12 levels (Chapter 3).
- To investigate and characterize the *Hp*-specific peripheral blood Th17 response (Chapter 4).
- To compare IL-17 mRNA and protein levels in the gastric mucosa of *Hp*+ and uninfected patients and compare relative Th17 cytokine and Th1 cytokine levels (Chapter 5).
- To investigate frequencies of Th17 cells and other IL-17 producing cells in the gastric mucosa of *Hp*+ and uninfected patients (Chapter 6).
- To correlate IL-17/Th17 cell markers with gastric neutrophil and lymphocyte infiltration in *Hp*+ patients (Chapters 5 and 6).
- To see if IL-17/Th17 responses and incidence of *Hp*-related peptic ulcer disease or precancerous pathology are associated (Chapters 5 and 6).

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 DENDRITIC CELL (DC) AND T CELL RESPONSES TO *HP*

2.1.1 Isolation/Generation of DCs and Naive CD4+ T Cells

Monocyte-derived dendritic cells (MoDCs) were generated as previously described (Jackson et al.). Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from donor buffy coats by density gradient centrifugation over Histopaque 1077 (Sigma), then washed in PBS. Monocytes were purified by positive selection of $CD14^+$ cells using magnetic beads typically resulting in >95% purity. The monocytes were cultured in RPMI-1640 with 10% (v/v) FCS, 1% (v/v) L-Glutamine, GM-CSF (1000U/ml) and IL-4 (800U/ml). Fresh media was added after 3 days and the immature MoDCs harvested after 5 or 6 days.

100mls heparinised blood from each donor was used to obtain $CD1c^+$ myeloid DCs (MyDCs) and $CD4^+CD45RA^+$ T cells. First PBMCs were isolated, as above. MyDCs were isolated using Miltenyi CD1c MyDC kit, as per the manufacturer's instructions. $CD19^+$ cells were depleted prior to positive selection of $CD1c^+$ cells, as a subset of B cells express CD1c. Resulting $CD1c^+$ populations were typically >95% pure.

Naive $CD4^+$ T cells for co-culture experiments were generated by $CD4^+$, followed by $CD45RA^+$ positive selection, again using magnetic beads.

Flow cytometry was used to assess phenotype and activation marker status on the MoDCs after 24 hours of culture with *Hp* at multiplicities of infection of 0, 5, 10, 20 and 50. Antibodies were purchased from Beckman Coulter unless otherwise specified: CD14-FITC (AbD Serotec) and CD80-PE or CD11c-PE or CD86-PE and HLA-DR-ECD and CD83-PC5 were used for extracellular staining. The cells were fixed with 0.5% paraformaldehyde (Biolegend, London, UK) and data acquired on an FC500 Flow cytometer (Beckman Coulter). Appropriate isotype and minus one controls were used to confirm that staining was specific. The data was analyzed using Weasel 2.5 Flow Cytometry Software.

2.1.2 Coculture of DCs with *Hp*

The AB21, AB31, A101 and 93-67 wild-type *Hp* strains from our group's international strain collection and their isogenic Δ*dupA* mutants, previously described in Hussein *et al.* (Hussein et al., 2010) were used. Isogenic Δ*cagE* and Δ*cagE*Δ*dupA* double mutants were also available for the *Hp* AB21 strain. The *Hp* strains were grown on DENT *Hp*-selective plates (containing trimethoprim, vancomycin, cefsulodin and amphotericin B). The bacteria were suspended in DC culture media and the OD_{600} used to estimate their concentration based on an estimate that an OD of 1.0 contains 5 X 10^8 *H. pylori* per ml. 1 x 10^4 DCs per well were plated in sterile flat-bottomed 96-well nunclon delta surface plates (Thermo Scientific). *Hp* was added at a multiplicity of infection (MOI) of 10, except where stated in the initial optimization experiments. A dilution series of each bacterial suspension was plated out to determine the number of colony forming units (CFUs). *E. coli* LPS (serotype O55:B50)(Sigma) 100ng/ml was used as a control. The DCs and *Hp* were co-cultured for 24 or 48 hours before harvesting supernatants.

2.1.3 Cytokine ELISAs to Assess DC Cytokine Secretion

eBioscience Ready-SET-Go! ELISA kits were used to quantify IL-1β, IL-6, IL-10, IL-12p70 and IL-23 levels in the DC supernatants. Human IL-8 CytoSet™ from Biosource was used for IL-8 quantification and ELISA MAX™ Human IL-12/IL-23 (p40) (BioLegend) for IL-12p40 quantification. All kits were used as per manufacturer's instructions except half volumes of all reagents and samples were used. A standard curve (in duplicate), 6 "blank" wells with assay diluents instead of sample and wells without streptavidin-peroxidase and secondary antibody were included on each plate. Samples were run in duplicate and diluted 1:10 for IL-1β and IL-23, 1:20 for IL-10, 1:200 for IL-12p40, 1:400 for IL-6 and IL-8 and 1:800 for IL-12p70. Plates were read on a microplate reader (LabSystems iEMS reader MF) at 450 and 595nm and the cytokine concentrations calculated using the local standard curve. The limit of sensitivity was calculated from the mean + 3 standard deviations of the "blank" control wells. Typical sensitivities were IL-1β 7.5 pg/ml, IL-6 7.2 pg/ml,

IL-8 7.8 pg/ml, IL-10 3.1 pg/ml, IL-12p40 41.0 pg/ml, IL-12p70 3.7 pg/ml, IL-23 10.6 pg/ml. Results were adjusted for the number of CFUs.

2.1.4. Coculture of T Cells with DC Supernatants and DCs

DC supernatants were filtered to remove any bacterial debris and added to naive $CD4^+$ T cells (1×10^5 per well) with anti-CD3/anti-CD28 Dynabeads® (Invitrogen) in sterile 96 well tissue culture plates in RPMI-1640 with 10% foetal calf serum, 1% HEPES, 1% sodium pyruvate, 1% nonessential aminoacids, 1% L-glutamine + 20μM 2-mercaptoethanol. Supernatants were harvested after 5 days and frozen at -80°C until analysis.

For co-culture of naive $CD4^+$ T cells a 1:10 DC to T cell ratio was used in 200 μl media in sterile 96 well tissue culture plates, as above and supernatants were harvested after 5 days. eBioscience Ready-SET-Go! ELISA kits were used to quantify IFNγ and IL-17 levels in the supernatants. These ELISAs were run and analyzed in the same manner as the ELISAs described above. Typical sensitivities were 45.7 pg/ml for IFNγ, 9 pg/ml for IL-17.

2.2 VOLUNTEERS AND CLINICAL MATERIALS

The study was approved by Nottingham Research Ethics Committee 2. Samples were donated by patients undergoing upper gastrointestinal endoscopy at the Queen's Medical Centre Hospital, Nottingham, with informed consent. None of the patients had been taking proton pump inhibitors in the preceding two weeks, antibiotics in the preceding 4 weeks or regular non-steroidal anti-inflammatory drugs.

Patients donated a blood sample and a series of gastric biopsies: One was placed in a rapid urease test (0.5 g urea + 100 μl (w/v) phenol red made up to 10 mls with distilled water) for an early indication of *Hp* status, one in iso-sensitest broth (Oxoid, Cambridge, UK)/10% glycerol for isolation and culture of *Hp,* biopsies from the antrum and corpus were placed in formalin for histopathology, one biopsy from the antrum was placed in RNAlater (Qiagen, Crawley, UK) and 6 further antral biopsies in culture medium (RPMI-1640 with L-glutamine, 2% FCS and 1% Antibiotic Antimycotic Solution (Sigma) for

analysis by flow cytometry. The rapid urease test was considered positive if the colour changed within 10 minutes and after this time the biopsy was removed from the test solution and frozen in liquid nitrogen for protein analysis. *Hp* DNA was extracted from cultured isolates and PCR genotyping carried out to ascertain *cagA* and *dupA* status, as previously described (Aviles-Jimenez et al., 2004, Hussein et al., 2010).

2.3 QUANTIFICATION OF GASTRIC CYTOKINE RESPONSES TO *HP*

2.3.1 ELISA

For IL-17 ELISA, biopsies were weighed, then homogenized in 250 µl sterile PBS using a mini pellet pestle (Kimble Kontes, NJ, USA) and centrifuged. Supernatants were assayed with eBioscience human IL-17 ELISA Ready-Set-Go! kit, as per the manufacturer's instructions, with an overnight incubation of the samples on the plate to maximize sensitivity. A standard curve and controls were included on each plate and plates were read at 450 and 595nm on a microplate reader, as above. The results were adjusted for biopsy weight.

2.3.2 Luminex

2.3.2.1 Kit comparison

To assess accuracy and determine the lower limit of quantitation of IL-17 and IFNγ for each kit, single biopsies from each patient were individually thawed and immediately disrupted in extraction buffer (A): RPMI-1640 (Sigma, Poole, UK) supplemented with 10% (v/v) fetal calf serum (Sigma, Poole, UK) and protease inhibitors (cOmplete mini [EDTA-free], Roche, Germany) with a mini pellet pestle (Kimble Kontes, NJ, USA) for 2 minutes. After extraction, supernatants were obtained by centrifugation at 10,000 x *g* for 10 minutes at 4°C.

9 biopsies each from 3 patients were individually prepared. 50µL of each resulting supernatant were combined for each patient (to give a total volume

of 450µL), split into three aliquots (to test with the 3 kits), and spiked with 15µL of known concentrations of recombinant human IL-17 and IFNγ (eBioscience) diluted in extraction buffer (A). Cytokine spikes were at final concentrations of 0, 0.75, 1.5, 6, 50, 100 and 1000pg/mL.

Biopsies from a further 4 patients were used for repeatability studies and optimization of processing methods. Biopsies were processed using methods (1): manually with a mini pellet pestle (Kimble Kontes, NJ, USA) for 2 minutes, as above; (2): a proportion of those disrupted by pestle were further homogenised by 5-10 repeated passes through a 23G needle and 1mL syringe, or (3): automatically with a bead-basher (TissueLyser LT, Qiagen), using a single 5mm stainless steel bead per sample at 50Hz for 3 minutes, in PBS-based extraction buffer (B): Phosphate-buffered saline (PBS, pH 7.4, Dulbecco A, Oxoid, Cambridge, UK), or (C): PBS supplemented with 2mM Mg^{2+} (Sigma, Poole, UK) and Benzonase® nuclease (25U/mL, Novagen, Germany), each supplemented with protease inhibitors, as above. Biopsies from 3 patients were disrupted in 600 µl extraction buffer and 4 identical aliquots prepared from each, which were included at different positions on the same plate, and a % coefficient of variation (%CV) calculated for each (mean/SD x 100). For tissue preparation comparisons 10 biopsies from 3 patients were spiked prior to biopsy disruption at 100pg/mL in the same total volume of extraction buffer as 10 paired, unspiked biopsies. Cytokine recovery was adjusted for background cytokine concentrations from the unspiked samples, and cytokine recovery using the different processing methods was compared.

Assays were run according to each manufacturer's instructions, with the exception that MilliPLEX beads were vigorously vortexed rather than using an ultrasonic bath. The VersaMAP and BioPLEX kits had non-magnetic beads and the MilliPLEX kit magnetic beads. Filter plates and vacuum washing was used for all three kits. Standard curves were run in duplicate as provided and recommended by each manufacturer but extended down to ≤0.5 pg/ml to assess sensitivity. Data were acquired on a Bio-Plex 200 system and analyzed with Bio-Plex Manager 6.0 software (both Bio-Rad (Laboratories), CA, USA).

Standards curves were plated in duplicate. Wells with bead counts <37 were excluded and points with coefficient of variation >25% were excluded. Lower and upper limits of quantitation were automatically calculated by the software (using 5 parameter logistic non-linear regression model, after excluding points on the standard curve with recovery outside of 80-120% of expected). The linear dynamic range (LDR) was defined as the lowest and highest standards on the linear part of each standard curve on a log-log plot.

2.3.2.2 Final Luminex Method

Samples were individually thawed, and disrupted in 300 µl PBS supplemented with 2mM Mg^{2+} (Sigma, Poole, UK), Benzonase® nuclease (25U/mL, Novagen, Germany), and protease inhibitors (cOmplete mini [EDTA-free], Roche, Germany) on ice, using pellet pestles with a cordless motor for 2 minutes, then homogenized by passing through a 200 µl filter tip (diameter equivalent to 21-22G syringe) at least 20 times. Homogenized samples were left on ice for at least 5 minutes (to allow the benzonase to work), then centrifuged at 10,000g for 10 minutes at 4°C, aliquoted into LoBind tubes (Eppendorf) and stored at -80°C until analysis.

Samples were analyzed using MILLIPLEX MAP Human Th17 Magnetic Bead Panel and MILLIPLEX MAP High Sensitivity Human Cytokine Magnetic Bead Panels, as per manufacturer's instructions, using a magnetic block for washing.

Pierce bicinchoninic acid (BCA) (Thermoscientific) was used to assay total protein content of an aliquot from each sample and results were adjusted accordingly.

2.4 IL-17 AND RORC2 RT-qPCR

RNA was extracted using RNeasy Mini Kits (Qiagen, Crawley, UK), as per kit instructions. The RNA was treated with Ambion® TURBO™ DNase (Life Technologies, Paisley, UK) to remove any contaminating genomic DNA and reverse transcribed to cDNA using random primers (Invitrogen) and Superscript II (Invitrogen). RT-qPCR was performed on a Rotor-Gene 3000

(Corbett Research, Cambridge, UK) using a DyNAmo HS SYBR Green Kit (New England BioLabs, Hitchin, UK). Amplification was carried out over 40 cycles of 15 seconds at 95°C, 30 seconds at 62°C and 30 seconds at 72°C. Samples were run in triplicate and no template controls and samples produced without reverse transcriptase were included in each run as negative controls and melting curve analyses were performed. Results were analyzed according to the Pflaffl method (Pfaffl, 2001). Relative gene expression levels were determined by normalizing against *GAPDH* and comparing to a negative comparator generated by pooling *Hp* negative samples. Commercial human cDNA (Clontech) was included in all assays as a positive control. Efficiencies were as follows: *RORC2* 1.59, *IL17A* 1.95, *IFNG* 1.95 (kindly analyzed by Dr R Ingram) and *GAPDH* 1.69.

Commercial IL17A primers were used (Qiagen, Crawley, UK). *RORC2* and *GAPDH* primer sequences are shown in Table 2.1 below.

Gene	Forward primer	Reverse primer
RORC2	CAGTCATGAGAACACAAATTG AAGTG (Burgler et al., 2009)	GGAAGAAGCCCTTGCACC
GAPDH (Walker et al., 2003)	CCACATCGCCAGACACCACT	GGCAACAATATCCACTTTACCA GAGT

Table 2.1 *RORC2* and *GAPDH* primer sequences

2.5 FLOW CYTOMETRY

2.5.2 Cell Isolation and Culture

Blood samples were collected into vacutainer tubes containing EDTA. Blood was centrifuged to separate plasma, then layered over Histopaque 1077

(Sigma, Poole, UK) to isolate PBMCs, which were washed 3 times, then counted and placed in culture medium (RPMI-1640 with 10% FCS, 1% L-glutamine or IMDM with 10% FCS, 1% L-glutamine) at a final concentration of 1 x 10^6/ml for overnight culture. Cells were cultured with medium only (unstimulated control), 20 ng/ml phorbol myristate acetate (PMA) and 1 µmol/l ionomycin (positive control), *Hp* whole cell sonicated antigen, final concentration 25 µg/ml (a mixture of 6 clinical strains isolated from Nottingham patients), tetanus toxoid (National Institute for Biological Standards and Control) at 5 Lfu/ml or *Candida albicans* (kindly donated by Immunology Department, Nottingham University Hospitals), final concentration 1 x 10^5/ml.

Gastric biopsies were rubbed through sterile disposable 100 µm cell strainers (BD Biosciences, Oxford, UK), washed and resuspended in culture medium (IMDM with 10% FCS, 1% L-glutamine). In most cases this yielded <2 x 10^6 cells. An unstimulated and a PMA/ionomycin (concentrations as above) stimulated sample was set up for each patient.

PBMC and gastric biopsy samples were stimulated for 16 hours with the addition of Brefeldin A (10 µg/ml) after 1 hour.

Some frozen PBMCs were used. They were prepared as above and placed in freezing medium (10% dimethyl sulphoxide (Sigma), 50% FCS, and 40% RPMI 1640), after washing, packaged in polystyrene to slow the rate of temperature drop and frozen at -80°C. To use they were rapidly thawed with addition of warm cell culture media, taking care to avoid osmotic shock, washed and counted (viability 72-88%) then resuspended at 1 x 10^6/ml for overnight incubation as above (Disis et al., 2006).

2.5.3 Extracellular and Intracellular Staining

PBMCs were stained with anti-CD4-PE-Texas red (ECD) (Beckman Coulter, High Wycombe, UK), fixed with 0.5% paraformaldehyde (Biolegend), washed, permeabilized and stained with anti-CD69-PC5 (Beckman Coulter) or anti-

CD154-PC5 (Biolegend), anti-IFNγ-FITC (Beckman Coulter) and anti-IL-17-PE (eBioscience).

Gastric biopsies were stained with anti-CD3-PC5, anti-CD4-PE-Texas red (ECD), anti-CD8-FITC (all from Coulter), washed, permeabilized and stained with anti-IL-17-PE (eBioscience).

Data were acquired on an FC500 Flow cytometer (Beckman Coulter). Appropriate isotype and minus one controls were used to confirm that staining was specific. The data was analyzed using Weasel 2.5 Flow Cytometry Software.

2.6 STATISTICAL ANALYSIS

Statistical analyses and box-and-whisker plots were prepared using GraphPad Prism 5 software. Boxes represent the 25^{th} to 75^{th} centiles, horizontal lines within the boxes represent the median and the whiskers depict the 5^{th} and 95^{th} centiles. Bar charts in Chapter 3 and Figures 4.2 and 4.3 were prepared using Microsoft Office Excel 2007. Figure 4.4 was prepared using MINITAB release 14 software.

The Mann-Whitney U-test was used for analysis of unpaired data and the Wilcoxon signed rank test for paired sets of data. Kruskal-Wallis was used where there were 3 or more groups. Correlations were tested for using Spearman's rank correlation test. A significant difference was taken as $p \leq 0.05$.

CHAPTER 3

INFLUENCE OF *HP* ON DENDRITIC CELLS AND T CELL DIFFERENTIATION

3. INFLUENCE OF *HP* ON DENDRITIC CELLS AND T CELL DIFFERENTIATION

3.1 INTRODUCTION

3.1.1 Dendritic Cells Present Antigen to T Cells

Dendritic cells (DCs) have a key role in determining what type of T helper response the host will mount to a pathogen. In order for the adaptive immune system to respond to a pathogen, the pathogen must be taken up by antigen presenting cells and presented, with co-stimulation to T cells. DCs are professional antigen presenting cells adapted for this process. The name DC comes from the immature DC, which has highly mobile processes known as dendrites and is phagocytic. Antigen is taken up at the site of infection. This causes the DC to mature, upregulating MHC class I and II (also known as HLA-DR) on which it can present antigen to $CD8^+$ and $CD4^+$ T cells respectively, and a number of co-stimulatory surface molecules, such as CD83, CD80 and CD86 (also known as B7-1 and B7-2) (see Figure 3.1)(Kapsenberg, 2003, Sallusto and Lanzavecchia, 2002).

If antigen is presented to a T cell (signal 1) without costimulation (signal 2) the T cell may become anergic, leading to tolerance. Upregulation of the chemokine receptor CCR7 facilitates trafficking of the DC loaded with antigen from the invading pathogen towards its ligands, CCL19 (MIP-3β) and CCL21 in organized lymphoid tissue (Hansson et al., 2006) where the antigen is presented to T cells, along with co-stimulatory signals. Factors including the strength of the T cell receptor-antigen interaction, strength of costimulation, cytokines and other factors in the local environment and type of antigen presenting cell determine which type of cell the naïve T cell will develop into (Kapsenberg, 2003, Shortman and Liu, 2002).

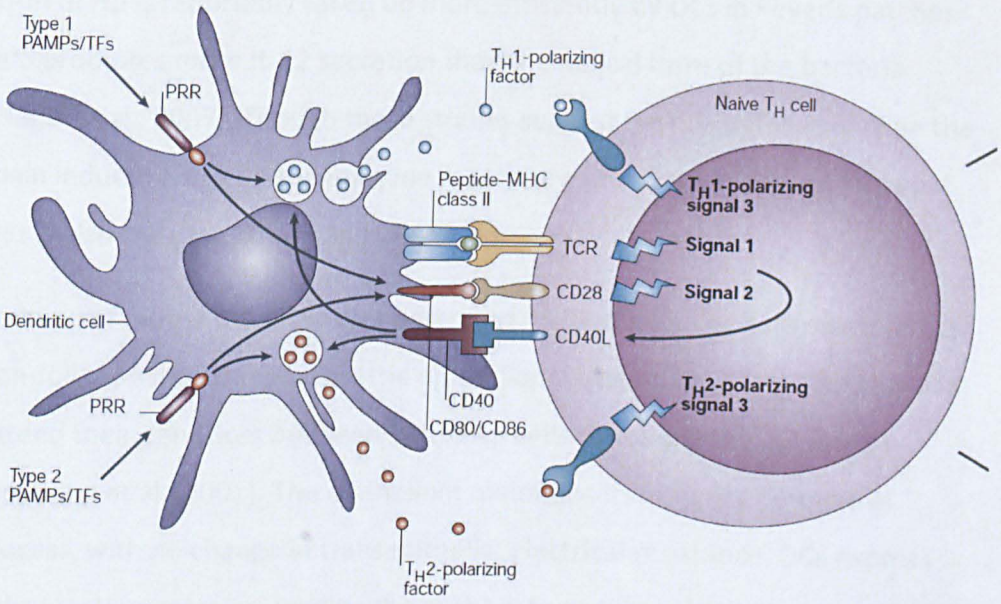


Figure 3.1 Naïve CD4$^+$ T cell stimulation and polarization requires three signals from the dendritic cell, adapated from Kapsenberg (Kapsenberg, 2003). Signal 1 is the presentation of pathogen derived antigen in the context of MHC class II. Signal 2 is the co-stimulatory signal, mainly delivered by stimulation of CD28 by CD80 and CD86 that are upregulated on the activated DC following recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Signal 3 is the polarizing signal, which can include cytokines and local tissue factors. CD40 ligand (CD40L) may be upregulated on the T cell following signal 1 and 2 and can then interact with CD40 on the DC to amplify the signal.

3.1.2 Uptake of Antigen for T Cell Priming in *Hp* Infection

In the intestine antigen can be taken up by specialized epithelial cells, called M cells, present in organized lymphoid tissue, such as Peyer's patches. During *Hp* infection small areas of organized lymphoid tissue known as lymphoid follicles can develop in the stomach which may act as an inductive site for the mucosal immune response. Evidence from studies using mice lacking Peyer's patches, however, suggests that these areas in the small intestine are important for development of *Hp*-associated gastritis, lymphocyte development and homing (Nagai et al., 2007, Kiriya et al., 2007). The coccoid

form of *Hp* is reportedly taken up more efficiently by DCs in Peyer's patches and promotes more IL-12 secretion than the helical form of the bacteria (Nagai et al., 2007). Though these studies suggest Peyer's patches may be the main inductive site for the immune response to *Hp* in mice it is not known if this is also the case in human *Hp* infection.

Staining of mouse intestine demonstrated further villous M cells scattered at non-follicle-associated sites in the epithelium (Jang et al., 2004). DCs can also extend their dendrites between epithelial cells to sample the gut lumen (Rescigno et al., 2001). The epithelium maintains its integrity during this process, with no change in transepithelial electrical resistance. DCs express tight-junction proteins, leading the authors to propose that tight junction-like structures are temporarily formed between DCs and epithelial cells (Rescigno et al., 2001). Dendrite extension is regulated by the fractalkine receptor (CX3CR1) (Niess et al., 2005). One report on *Hp*-infected mice used immunohistochemistry and electron microscopy to visualize transepithelial DC dendrites in inflammed *Hp*-infected gastric mucosa (Necchi et al., 2009). Bacterial toxins can damage the epithelial barrier, further facilitating interaction between host DCs and the invading bacteria, for example CagA can disrupt epithelial apical-junctional complexes (Amieva et al., 2003). Uptake of IgG-bound antigen may occur in an FcRn (neonatal Fc receptor for IgG)-dependent manner in the intestine (Yoshida et al., 2006), but this has not been reported for *Hp* antigens specifically.

3.1.3 Different Types of Dendritic Cell

Different types of DCs and their nomenclature can be confusing. Blood is the easiest compartment to study in humans but DCs do not become fully mature until they meet pathogen associated molecular patterns (PAMPs) and/or inflammatory cytokines in tissue when their phenotype radically changes (Huang et al., 2001). DC precursors in blood may be divided into myeloid and plasmacytoid subsets. The plasmacytoid subgroup are CD11c-, express the IL-3 receptor, respond to pathogens with IFNα/β production and tend to

promote a Th2-biased response (Shortman and Liu, 2002). A consensus nomenclature document published in 2010 recommends subdividing the myeloid group into CD1c+ and CD141+, a minor, IFNβ producing subset (Ziegler-Heitbrock et al., 2010). The three groups of blood DCs can be differentiated by antibodies to CD303 (BDCA-2) (plasmacytoid), CD1c (BDCA-1) and CD141 (BDCA-3) (Dzionek et al., 2000, Ziegler-Heitbrock et al., 2010) (Table 3.1).

Type of DC	Surface markers	Commercial antibody	Antibody specificity	Notes
Plasmacytoid	$CD11c^-$, $IL\text{-}3R^+$, $CD68^+$	BDCA-2	CD303	IFNα/β producers Promote Th2 bias
Myeloid	$CD11c^+$ $CD13^+$, $CD33^+$	BDCA-1	CD1c	CD1c also expressed on a proportion of B cells
		BDCA-3	CD141	Minor subset Produce IFNβ

Table 3.1 Subsets of DC precursors in human blood.

Tissue DCs phenotypes can vary depending on the organ. The first DC to be described was the Langerhans cell in skin with characteristic Birbeck granules. In the gastrointestinal tract DCs need to prime a protective response against pathogens yet tolerate commensal organisms and food. The intestine has been the focus of most gastrointestinal DC research and the vast majority of studies have been done in mice. There is a profound lack of data concerning human gastrointestinal DCs. A number of different types have been described but they can be broadly divided into those that express CD103 (receptor for E-cadherin) and those that do not. The $CD103^+$ mesenteric lymph node and lamina propria DCs promote Treg differentiation in a TGFβ and retinoic acid

dependent manner (Sun et al., 2007, Coombes et al., 2007). DCs express aldh1a2, a retinal dehydrogenase that can convert retinal into retinoic acid (Coombes et al., 2007). $CD103^+$ intestinal DCs also have retinoic acid dependent roles in imprinting T and B cells with $\alpha4\beta7$ (ligand MadCAM-1) and CCR9 (ligand TECK/CCL25) to facilitate gut homing (Mora et al., 2003, Iwata et al., 2004, Johansson-Lindbom et al., 2005) and in B cell class switching to IgA (Mora et al., 2006).

3.1.4 Stimulation of Dendritic Cells by *Hp*

C57BL/6 mice recruited $CD11c^+$ DCs to their gastric mucosa as soon as 6 hours after *Hp* infection and studies with DCs expressing yellow fluorescent protein indicate that they move nearer to the epithelial surface (Kao et al., 2006b, Kao et al., 2010). Dendritic cells ($HLA\text{-}DR^+CD11c^+$) have been demonstrated within the human gastric mucosa and are more numerous in subjects with *Hp* infection (Bimczok et al., Oertli et al., 2012). These cells have only been characterized in small numbers of patients and there is controversy as to whether *Hp* infection increases activation of DCs *in vivo*, with conflicting results from different groups (see discussion) (Bimczok et al., Oertli et al., 2012). Insufficient co-stimulation may lead to an anergic T cell response and could help persistence of infection, but *Hp* is known to induce a $CD4^+$ T cell response, although this is ineffective at clearing the infection. Myeloid ($CD1c^+$) DCs were found in gastric mucosal biopsies from *Hp*-infected but not uninfected patients (Khamri et al., 2010).

Hp can interact with DCs in a number of different ways. Studies using $Myd88^{-/-}$ mice indicate that toll-like receptors (TLRs) have a role in alerting DCs to the presence of the bacteria, though *Hp* TLR ligands are less potent than those of many other enteric bacteria (Lee and Josenhans, 2005, Gewirtz et al., 2004). DCs from $Myd88^{-/-}$ mice upregulated activation markers to a lesser extent and produced lower concentrations of IL-12 and IL-6 in response to *Hp* infection. Reduced gastritis and increased colonization densities were found in the $Myd88^{-/-}$ mice *in vivo*, in keeping with a reduced inflammatory response (Rad

et al., 2007). In a study using the *H. felis* infection model Myd88$^{-/-}$ mice were found to have reduced IFNγ, IL-17A, IL-22 responses to *Hp* and reduced expression of the antibacterial peptide lipocalin-2, but no significant reduction in inflammation scores was found in the null mice (Obonyo et al., 2011). Hafsi *et al.* found that polymyxin B did not significantly alter the IL-12 response of MoDCs to *Hp in vitro*, suggesting interaction of *Hp* LPS with TLR4 does not make a major contribution (Hafsi et al., 2004). Conversely Kranzer *et al.* found addition of polymyxin B reduced IL-6 and IL-8 secretion (Kranzer et al., 2005). Rad *et al.* have subsequently identified a number of different pathways by which DCs can recognize *Hp* in a study using a range of TLR knockout mice. They report TLR2 to be the most important extracellular TLR, triggering an anti-inflammatory response, with DCs from TLR2$^{-/-}$ having an abrogated IL-10 response (Rad et al., 2009). A TLR2 dependent IL-10 secretion pathway has also been described in murine bone marrow derived dendritic cells (BMDCs) (Wang et al., 2010). *Hp* upregulated TLR2 expression in murine BMDCs more than exposure to *E. coli* or the gastric pathogen *Acinetobacter lwoffi*, and *Hp*-stimulated BMDCs were able to convert naïve T cells into Tregs (Zhang et al., 2010b). However a study using PBMCs suggests a more pro-inflammatory role for TLR2 in humans as TLR2 stimulation was able to convert both naïve and effector Tregs to Th17 cells (Nyirenda et al., 2011). The *Hp* neutrophil-activating protein also mediates pro-inflammatory effects on human neutrophils and monocytes through TLR2 (Amedei et al., 2006).

Different types of DC and pre-DC express different TLRs. PCR studies showed that monocytes express TLR 1, 2, 4, 5, and 8, and low level of TLR6, whereas CD11c^{+} immature DCs expressed predominantly TLR 1, 2 and 3 and plasmacytoid pre-DCs showed strong expression of TLR 7 and 9 (Kadowaki et al., 2001).

Gastric DCs internalize *Hp* (Bimczok et al.), therefore cytosolic Pattern Recognition Receptors (PRRs) such as intracellular TLRs and NOD receptors could also have a role. TLR9 can bind *Hp* DNA causing a proinflammatory IL-12p40 and IL-6 response (Rad et al., 2009). NOD1 is known to recognize

peptidoglycan delivered to gastric epithelial cells by the *Hp* type IV secretion system encoded by the *cag* pathogenicity island (Viala et al., 2004) and work with gastric epithelial cells lines indicates NOD1 is an important sensor for expression of the antibacterial peptide human β-defensin-2 (Grubman et al., 2010, Boughan et al., 2006). NOD2 stimulation is known to be able to promote IL-17 secretion by memory T cells (van Beelen et al., 2007) but there is little in the literature on stimulation of NOD1 or NOD2 in DCs by *Hp*. A number of small studies have looked for associations between NOD2 mutations and *Hp*-related disease, with particular interest in gastric lymphoma, but the results have been conflicting (Rosenstiel et al., 2006, Ture-Ozdemir et al., 2008)

A number of other components of *Hp* can interact with host immune cells to influence T cell differentiation. *Hp* urease upregulated HLA-DR and IL-2R on monocytes and increased their production of IL-1β, IL-6, IL-8 and TNFα (Harris et al., 1996). Mice vaccinated with recombinant urease subunit B had an enhanced Th17 response (subcutaneous and intranasal routes) with reduced *Hp* colonization (intranasal route only) (Zhang et al., 2011). The *Hp* neutrophil-activating protein induces IL-12 and IL-23 expression and upregulates HLA-DR, CD80 and CD86 on monocytes and promotes IFNγ producing T cells (Amedei et al., 2006). LPS Lewis antigens on *Hp* are able to bind DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) on DCs (Appelmelk et al., 2003), which increases DC IL-10 production and reduces T cell IFNγ production (Bergman et al., 2004). *Hp* can switch Lewis antigen expression on and off using translational frameshifting (phase-variable expression), thereby altering the balance of Th1/Th2 cells it induces (Bergman et al., 2004). The *Hp* plasticity region locus *jhp0947–jhp0949*, which is associated with duodenal ulcer disease, caused increased IL-12 secretion by the THP-1 cell line (de Jonge et al., 2004).

3.1.5 Dendritic Cell Cytokine Responses to *Hp* Stimulation

A number of different models of human DCs have been used in *in vitro* studies but the most commonly used in the *Hp* field, is isolation of $CD14^+$ cells from peripheral blood and culture with IL-4 and GM-CSF for 5-6 days to obtain immature monocyte derived DCs (MoDCs) (Sallusto and Lanzavecchia, 1994). Other models have used $CD34^+$ precursors from umbilical cord blood or bone marrow cultured with cytokines (Shortman and Liu, 2002). In an alternative strategy described by Randolph *et al.* blood monocytes are incubated with an endothelial cell layer (derived from umbilical vein) over a collagen matrix. The cells migrate into the matrix (analogous to cells migrating into tissue), then a proportion transmigrate back, said to represent trafficking from the tissue to lymph node. No cytokines are required and DCs are obtained in only 2 days (Randolph et al., 1998) but this model has not been widely adopted. Leukaemia derived DC cell lines, such as THP-1 are available but do not mimic DCs in all respects and may develop more macrophage-like features under some conditions (Santegoets et al., 2008). BMDCs are commonly used in murine studies.

The relative balance of IL-12 family cytokines produced by *Hp*-stimulated DCs could indicate whether *Hp* predominantly primes a Th1 or Th17 response. IL-12 is well established as a pro-Th1 cytokine and IL-23 promotes Th17 expansion and terminal differentiation (McGeachy et al., 2009). The p40 subunit, common to IL-12 and IL-23 was produced in much greater amounts than IL-12p70 or IL-23 by PBMCs stimulated by *Hp*. Stimulation using dupA-deficient *Hp* mutant strains led to a reduction in secretion of p40, IL-12p70 and IL-23 (Hussein et al., 2010). The IL-12p70 response of MoDCs to *Hp* has been studied by a number of groups. Most authors found that *Hp*-stimulated MoDCs produced large amounts of IL-12p70 (Guiney et al., 2003, Kranzer et al., 2004, Hafsi et al., 2004, Bimczok et al.). However others including Khamri *et al.* have found a low IL-12p70 human DC response to *Hp*. Mitchell *et al.* found very little IL-12p70 production unless CD40L was added (Mitchell et al., 2007). In a study using murine BMDCs *Hp*-stimulated DCs secreted less IL-

12p70 than DCs stimulated by *Acinetobacter lwoffi* and *Hp* sonicate appeared to block IL-12 release by *A. lwoffi* stimulated DCs (Kao et al., 2006b). Oertli *et al.* found low CD80 and CD86 on DCs from *Hp*-infected human gastric tissue suggesting that DCs in the *Hp*-infected mucosa may not fully mature and may therefore be inefficient at priming pro-inflammatory responses (Oertli et al., 2012).

IL-23 has attracted less interest but was found at higher levels than IL-12p70 in one study (Khamri et al., 2010). Confocal microscopy demonstrated co-localization of IL-23 with CD1c, suggesting it may be produced by myeloid DCs (Khamri et al., 2010).

IL-8 has been a key cytokine studied in *Hp* research for many years. It is produced in large amounts by epithelial cells upon *Hp* stimulation and has an important role in recruiting neutrophils to the infected mucosa. DCs can also produce large amounts of IL-8 in response to *Hp* (Bimczok et al., Hafsi et al., 2004, Kranzer et al., 2004, Kranzer et al., 2005). There was a small but significant reduction in IL-8 when PBMCs were stimulated with Δ*dupA* mutants compared to wild type *Hp* strains (Hussein et al., 2010). *Hp* strains with the *cag*PAI virulence factor are well recognized to induce more IL-8 secretion by gastric epithelial cells (Sharma et al., 1995).

IL-1β and IL-6 are both proinflammatory cytokines involved in human Th17 differentiation (Acosta-Rodriguez et al., 2007a). IL-6 can be produced by *Hp*-stimulated MoDCs (Kranzer et al., 2004, Kranzer et al., 2005) and gastric DCs (Bimczok et al.) but in one study IL-6 was secreted by *Salmonella*-stimulated but not *Hp*-stimulated MoDCs (Guiney et al., 2003). Other sources of IL-6 include gastric epithelial cells and macrophages. MoDCs also secrete IL-1β upon *Hp* stimulation (Kranzer et al., 2005). Caspase-1 is activated by *Hp* and processes pro-IL-1β to the active form in *Hp* infected BMDCs (Hitzler et al., 2012b). However MoDCs and macrophages produce much less IL-1β than monocytes following stimulation with LPS, TLR2 ligand (Pam3Cys) or *Staphylococcus epidermidis* (Netea et al., 2009).

IL-10 is a key regulatory cytokine and DC-derived IL-10 has a role in the differentiation of Tr1 Tregs (Levings et al., 2005). IL-10 is secreted by MoDCs (Kranzer et al., 2004, Kranzer et al., 2005, Mitchell et al., 2007), BMDCs (Kao et al., 2006b) and gastric DCs (Bimczok et al.) upon *Hp* stimulation. Most authors have reported IL-10 levels to be lower than IL-12p70 levels (Guiney et al., 2003, Kranzer et al., 2004, Kranzer et al., 2005, Kao et al., 2006b) but Mitchell *et al.* found them to be higher (Mitchell et al., 2007).

3.1.6 Effect of *Hp* Virulence Factors on Dendritic Cell Responses

Two of the best characterized virulence factors of *Hp* are CagA and VacA. As described in section 1.1.5.1, CagA is encoded by one of approximately 30 genes that make up the *cag* (*cytotoxin-associated gene*) pathogenicity island (*cag*PAI). Genes in this region encode a type IV secretion system which transfers bacterial proteins, including CagA, into gastric epithelial cells. Here it has effects on multiple cellular processes including migration, proliferation and apoptosis (Blaser and Atherton, 2004). The *cagE* gene encodes an essential part of the type IV secretion system so without it CagA cannot mediate its effects. VacA is a polymorphic gene which classically causes vacuolation in gastric epithelial cell lines and forms hexameric pores in membranes *in vitro*. It also has some immunosuppressive effects including inhibition of antigen presentation (Molinari et al., 1998) and reduction of T cell proliferation (Gebert et al., 2003). VacA is discussed in detail in section 1.1.5.2.

Studies using isogenic *cagE* virulence factor mutants found that mutation of *cagE* reduced IL-12p70 (Guiney et al., 2003) and IL-23 (Khamri et al., 2010) secreted by *Hp*-stimulated MoDCs but a study using 8 wild type strains found no effect of *cag* pathogenicity island or VacA status on cytokine secretion (Kranzer et al., 2005). Andres *et al.* did not find any significant difference in secretion of a panel of cytokines, including IL-12p70, in 20 clinical isolates of *Hp* analyzed by *cagA*, *cag*PAI or *vacA* status. However

BMDCs infected with *Hp* strains deficient in the virulence factors γ-glutamyl transpeptidase or VacA had increased expression of CD80 and increased secretion of IL-12p40 (Oertli et al., 2013). Mice infected with the same *Hp* isogenic mutants had reduced Treg frequencies, lower *Hp* colonization densities and increased Th1 and Th17 responses, suggesting that these virulence factors are involved in driving Treg responses and immune tolerance (Oertli et al., 2013).

The virulence factor *dupA* (described in detail in section 1.1.5.3) was only described relatively recently (Lu et al., 2005). It is a *virB4* homologue, suggesting it may have a role translocating bacterial products into epithelial cells, analogous to *cagE*, but its function has not yet been clarified. Monocytes stimulated with *dupA* deficient mutant *Hp* strains produced less IL-12p40, IL-12p70 and IL-23 than the parental wild-type strain, with small but significant differences in IL-8 (Hussein et al., 2010) suggesting that this virulence factor may act on immune cells rather than epithelial cells. No studies on the effect of *dupA* on the response of DCs to *Hp* have been published to date.

3.2 AIMS

i. To establish the balance of Th1/Th17 differentiation promoted by *Hp*-stimulated DCs.

ii. To determine the effects of mutation of virulence factors *dupA* and *cagE* on secretion of a range of cytokines by *Hp*-stimulated DCs.

iii. To compare responses to *Hp* using two models for human myeloid DCs.

3.3 RESULTS

3.3.1 Optimization of Conditions for Co-Culture of Monocyte-Derived DCs with *Hp*

Hp was co-cultured with MoDCs in 96 well plates at a range of multiplicities of infection (MOIs) and for 2 different durations (24 and 48 hours) to select optimal conditions for further experiments. Other authors have used MOIs of 5-20 and 15-48 hour time points in similar studies (Guiney et al., 2003, Kranzer et al., 2004, Kranzer et al., 2005, Hafsi et al., 2004, Mitchell et al., 2007, Khamri et al., 2010, Bimczok et al., 2010). Concentrations of different cytokines may peak at different time points. It was necessary to select a suitable time point to assess IL-23 in view of its role in Th17 proliferation and maintenance.

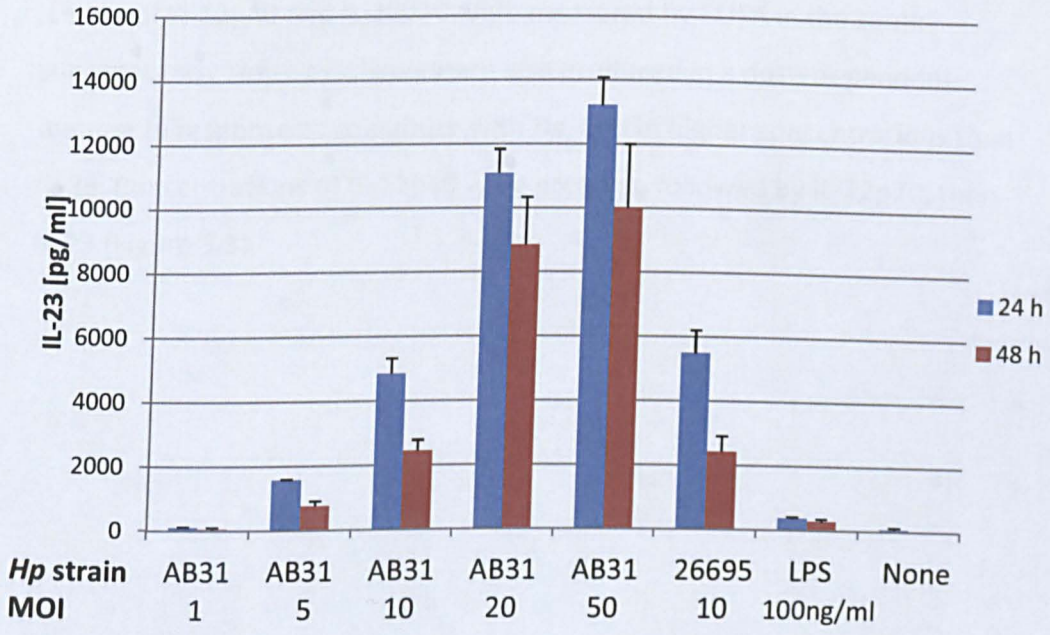


Figure 3.2 DCs produce IL-23 in a dose-dependent manner in response to *Hp*. MoDCs were co-cultured with a range of multiplicities of infection (MOI) of *Hp* or controls (LPS or medium only) and supernatants harvested after 24 and 48 hours. IL-23 levels were measured by ELISA. Bars represent mean of duplicate results for 3 replicate wells and error bars represent standard deviations.

The AB31 strain was used as an isogenic *dupA* mutant of this *Hp* strain was available and the effects of this virulence factor on *Hp*-primed DC cytokine production were to be assessed later. The 26695 strain of *Hp* was included in initial optimization experiments (Figures 3.2 and 3.3), for comparison, as it was the strain used in a similar study by Khamri *et al.* (Khamri et al., 2010). AB31 and 26695 strains are both *cag* PAI+, AB31 is *dupA*+ but 26695 is *dupA*-. LPS from *E. coli* (serotype O55:B50), also used by Khamri *et al.* was included as a positive control.

IL-23 production by MoDCs increased in a dose-dependent manner in response to co-culture with *Hp* strain AB31 (Figure 3.2). Increasing the incubation time from 24 to 48 hours did not increase the concentrations of cytokine therefore a 24 hour period was used for all subsequent experiments.

Levels of IL-12p40 and IL-12p70 were measured by ELISA in the same supernatants. These cytokines were also produced in a dose-dependent manner in response to co-culture with *Hp,* and in higher concentrations than IL-23. Concentrations of IL-12p40 were greatest, followed by IL-12p70, then IL-23 (Figure 3.3).

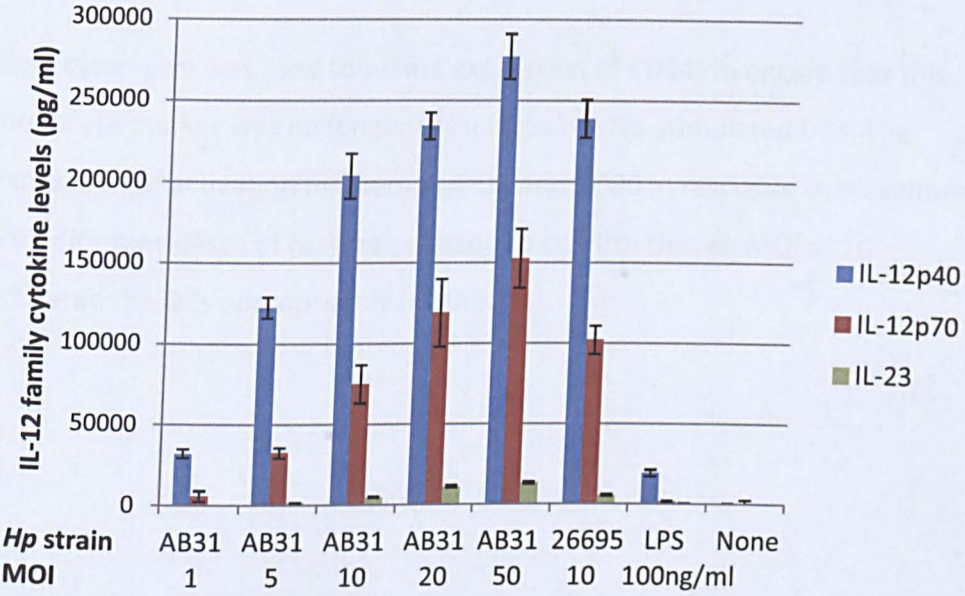


Figure 3.3 IL-12p40 and IL-12p70 were also produced in a dose-dependent manner in response to *Hp*, and in greater amounts than IL-23. MoDCs were co-cultured with *Hp* at a range of MOIs or controls (LPS and medium only) and supernatants harvested at 24 hours. Levels of p40, IL-12p70 and IL-23 in the supernatants were measured by ELISA. Bars represent mean of duplicate results for 3 replicate wells and error bars represent standard deviations.

LPS provoked much less IL-23, IL-12p70 and p40 (all $p<0.005$) secretion than *Hp* AB31 MOI 10. This has been noted previously for IL-12p70 and IL-1β by other authors using a panel of 8 different strains of *Hp* (Kranzer et al., 2004). Single TLR agonists such as LPS may not activate DCs fully without additional T cell signals, such as CD28 and CD40L binding (Figure 3.1). Pathogens may stimulate multiple TLRs, which can act synergistically in the absence of T cells (Napolitani et al., 2005). A further possibility is that the 24 hour time point was not optimal for LPS-stimulation of the cytokines measured.

The 26695 strain of *Hp* stimulated similar levels of cytokine secretion to the AB31 strain (Figures 3.2 and 3.3).

Flow cytometry was used to assess expression of CD14, to ensure that this monocyte marker was no longer present on the *Hp*-stimulated DCs. The expression of activation markers HLA-DR and CD80 in response to co-culture with different doses of *Hp* was assessed to confirm that an MOI of 10 activated the DCs appropriately (Figure 3.4).

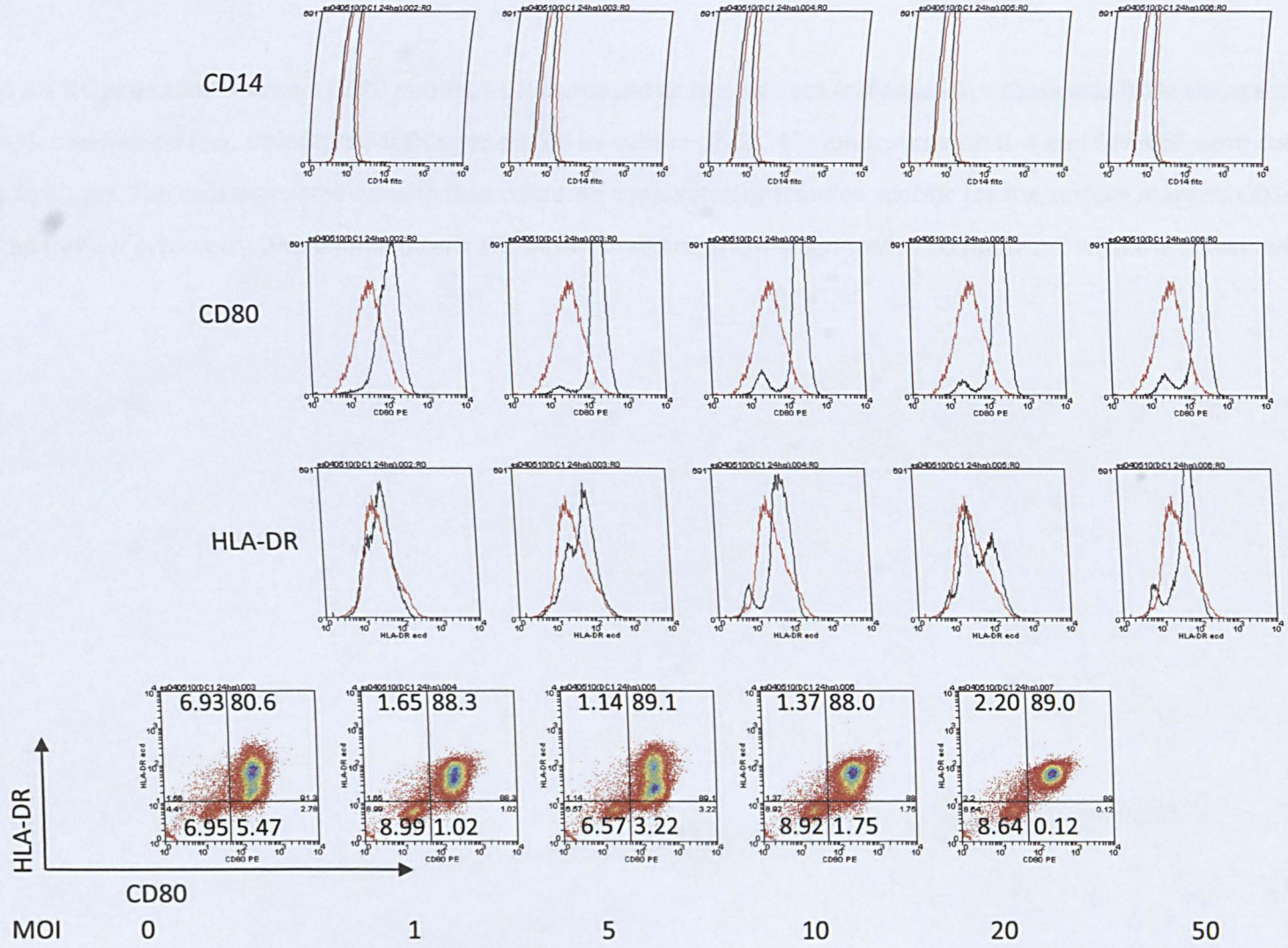

CD14
CD80
HLA-DR
HLA-DR
CD80
6.93 80.6
6.95 5.47
1.65 88.3
8.99 1.02
1.14 89.1
6.57 3.22
1.37 88.0
8.92 1.75
2.20 89.0
8.64 0.12
MOI
0
1
5
10
20
50

Figure 3.4 DC activation markers CD80 and HLA-DR increased as the Hp dose increased but there was little change above an MOI of 10. CD14 expression remained low. Immature MoDCs generated by culture of $CD14^+$ monocytes with IL-4 and GM-CSF were cultured with *Hp* for 24 hours, as above. The cells were stained with fluorochrome conjugated antibodies specific for the surface markers CD14, CD80 and HLA-DR and analyzed by flow cytometry.Unstimulated cells are shown in red on the histograms for comparison, with the *Hp*-stimulated MoDCs in black.

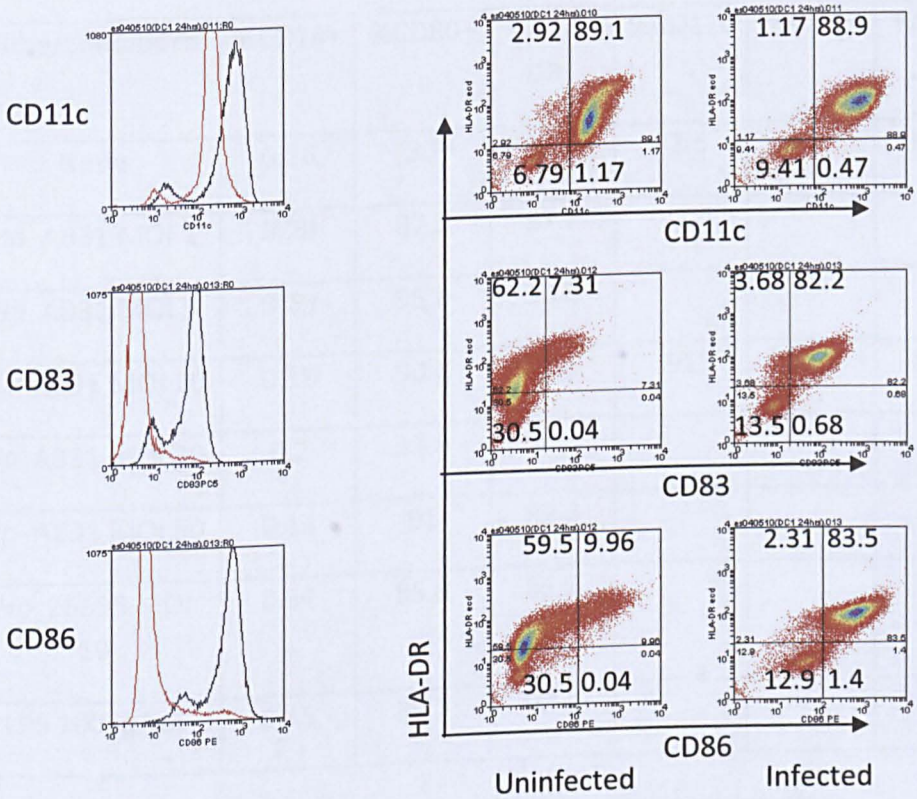


Figure 3.5 DC markers CD11c, CD83 and CD86 also increased following incubation with *Hp* strain AB31 at an MOI of 10. Immature MoDCs generated by culture of $CD14^{+}$ monocytes with IL-4 and GM-CSF were cultured with *Hp* for 24 hours, as above. The cells were stained with fluorochrome conjugated antibodies specific for the surface markers CD11c, CD83, CD86 and HLA-DR and analyzed by flow cytometry.

Tube/conditions	%CD14+	%CD80+	%HLA-DR+	%CD11c	%CD83	%CD86
None	0.26	30	88.4	92.5	4.38	6.32
Hp AB31 MOI 1	0.28	87.5	87.7			
Hp AB31 MOI 5	0.29	95.8	93.8			
Hp AB31 MOI 10	0.19	90.6	90.6	91	83.4	85.6
Hp AB31 MOI 20	0.2	93.8	90.9			
Hp AB31 MOI 50	0.13	91	89.8			
Hp 26695 MOI 10	0.34	85.8	86.2			
LPS 100ng/ml	0.15	89.2	90.1			

Table 3.2 Percentages of MoDC cell surface markers positive after stimulation with a range of MOIs of *Hp*. MoDCs were cultured with *Hp* (or medium only or LPS as controls) for 24 hours, then stained with fluorochrome conjugated antibodies. This data is shown in plots in Figures 3.4 and 3.5.

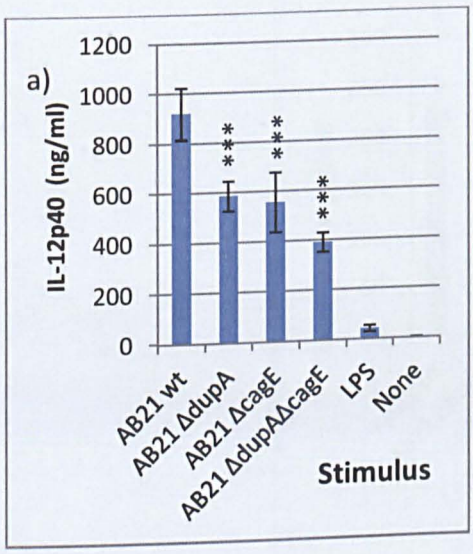
CD14 expression on the DCs was low, as expected, in all conditions (Figure 3.4, Table 3.2). No significant further increase in HLA-DR or CD80 expression was seen at MOIs greater than 10 (Figure 3.4 and Table 3.2). CD11c, CD83 and CD86 also increased following incubation with *Hp* strain AB31 at an MOI of 10 (Figure 3.5 and Table 3.2). All subsequent experiments were done with a MOI of 10.

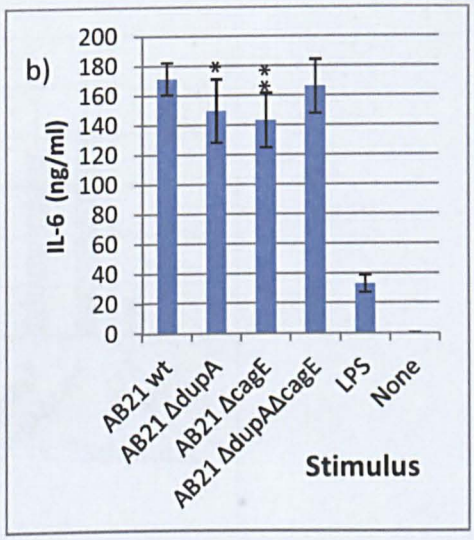
Others in the research group have shown that PBMCs exposed to *Hp* strains deficient in the virulence factor *dupA* produced less IL-12p40, IL-12p70 and IL-23. Small but significant differences in IL-8 were levels were also found (Hussein et al., 2010). There are more marked differences between antral IL-8 responses to *dupA*+ and *dupA*- strains *in vivo* (Lu et al., 2005), however monocytes are not present in the gastric mucosa. Flow cytometry suggested

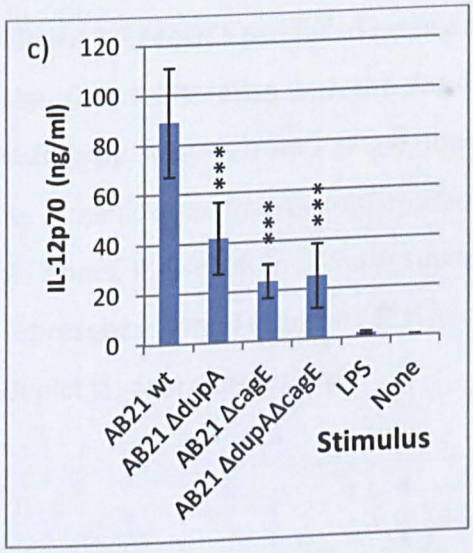
that the majority of cytokine producing PBMCs were $CD14^+$ monocytes (Hussein et al., 2010). We hypothesized that DCs could be the source of the *dupA*-induced differences *in vivo* and therefore cytokine production by MoDCs in response to *Hp in vitro* may be affected by *dupA* expression.

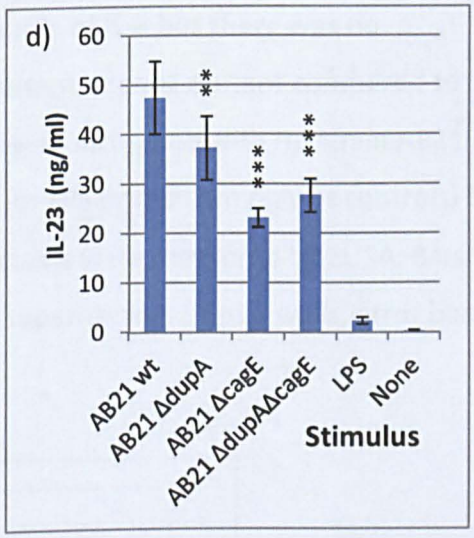
3.3.2 Effects of Mutating Virulence Factor Genes *dupA* and *cagE* on Cytokine Secretion by *Hp*-Stimulated MoDCs

Hp strain AB21 is *dupA*+ and *cag*PAI+. Isogenic *dupA*, *cagE* and a double *dupA* and *cagE* mutant were available for this strain. It has been reported by Khamri *et al.* that stimulation of MoDCs with an isogenic *cagE* mutant of *Hp* strain 84-183 led to lower concentrations of IL-23 than stimulation with wild type *Hp* 84-183. It was therefore desirable to include mutants of both these virulence factors in our study so that we could see which was having the greatest effect on cytokine secretion. The immature MoDCs were cultured with the different strains of *Hp* or controls in 96 well plates for 24 hours. The supernatants were then collected and cytokines analyzed by ELISA.









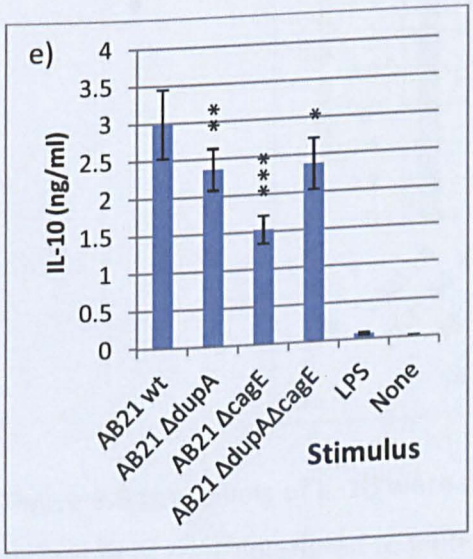


Figure 3.6 MoDCs incubated with *Hp* AB21Δ*dupA* or AB21Δ*cagE* produced less IL-12p40, IL-6, IL-12p70, IL-23 and IL-10 than those incubated with *Hp* AB21wt. MoDCs from a single donor were incubated with *Hp* strain AB21 or its isogenic *dupA* and/or *cagE* mutants (or LPS or medium only as controls) for 24 hours. Cytokine levels in 24 hour supernatants were measured by ELISA. (Graphs shown in order of prevalence of cytokines.) * indicates $p<0.05$, ** $p<0.005$ and *** $p<0.0005$. Bars represent mean of duplicate ELISAs on supernatants from 3 wells, error bars depict standard deviations.

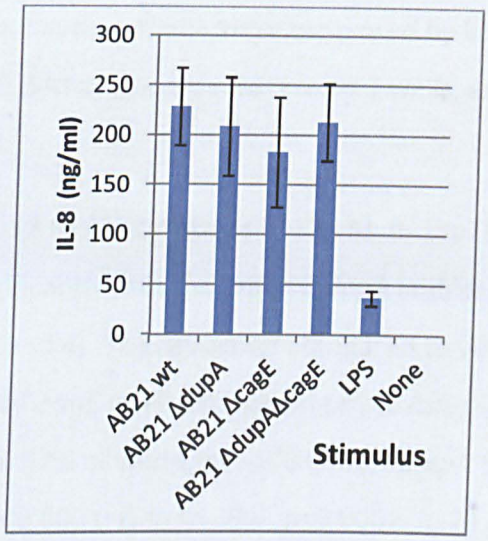


Figure 3.7 MoDCs produced large amounts of IL-8 but there was no significant difference with the *dupA* mutant or *cagE* mutant compared to wild-type. MoDCs from a single donor were incubated with *Hp* strain AB21 or its isogenic *dupA* and/or *cagE* mutants (or LPS or medium only as controls) for 24 hours. IL-8 levels in 24 hour supernatants were measured by ELISA. Bars represent mean of duplicate ELISAs on supernatants from 3 wells, error bars depict standard deviations.

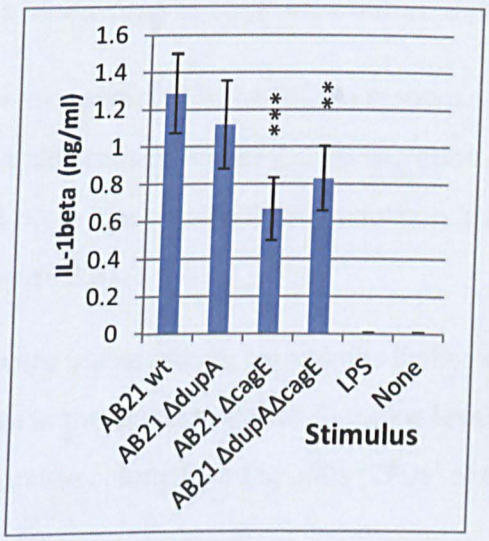


Figure 3.8 Low levels of IL-1β were produced. These were reduced with mutation of *cagE* compared to wild-type, but not with mutation of *dupA*. MoDCs from a single donor were incubated with *Hp* strain AB21 or its isogenic *dupA* and/or *cagE* mutants (or LPS or medium only as controls) for 24 hours.

IL-1β levels in 24 hour supernatants were measured by ELISA. Bars represent mean of duplicate ELISAs on supernatants from 3 wells, error bars depict standard deviations.

Production of the IL-12 family cytokines IL-12p40, IL-12p70 and IL-23 by MoDCs were all significantly reduced when Δ*dupA* and/or Δ*cagE* strains were used (Figure 3.6 a, c and d). The reduction compared to wild-type was more pronounced with the Δ*cagE* strain compared to the Δ*dupA* strain for all three cytokines: p40 Δ*cagE* 67% of wild-type, p<0.0001; Δ*dupA* 81%, p=0.0001, IL-12p70 Δ*cagE* 26%, p<0.0001; Δ*dupA* 39%, p=0.0002, IL-23 Δ*cagE* 48%, p=0.0001; Δ*dupA* 77%, p=0.0035.

IL-6 and IL-10 were also significantly reduced with the mutant strains compared to wild-type (Figure 3.6 b and e). Again absence of *cagE* resulted in a greater reduction of cytokine secretion than mutation of *dupA*: IL-6 Δ*cagE* 81% of wild-type, p<0.0024; Δ*dupA* 91%, p=0.018, IL-10 Δ*cagE* 54%, p=0.0002, Δ*dupA* 81%, p=0.0038.

MoDCs produced large amounts of IL-8 in response to *Hp,* but there was no significant reduction when *dupA* or *cagE* were mutated (Figure 3.7)

Low levels of IL-1β were produced by MoDCs in response to *Hp.* The Δ*cagE* and Δ*cagE* Δ*dupA* strains caused reduced IL-1β secretion compared to the wild-type strain, but there was no significant reduction in IL-1β secretion with the Δ*dupA* strain (Figure 3.8).

The cytokine levels were unexpectedly sometimes higher in the double, *dupA* and *cagE* mutant than in the single mutants. Cytokine levels were corrected for small differences in the colony forming units (CFUs) of the strains, so this is difficult to explain.

To confirm these results the experiment was repeated using MoDCs from more donors and a range of *Hp* strains, including two with and two without active *dupA* types. AB21, AB31, A101 and 93-67 *Hp* strains are all *cag* PAI+,

however AB21 and AB31 strains have active *dupA* secretion systems but A101 and 93-67 strains do not (Hussein et al., 2010).

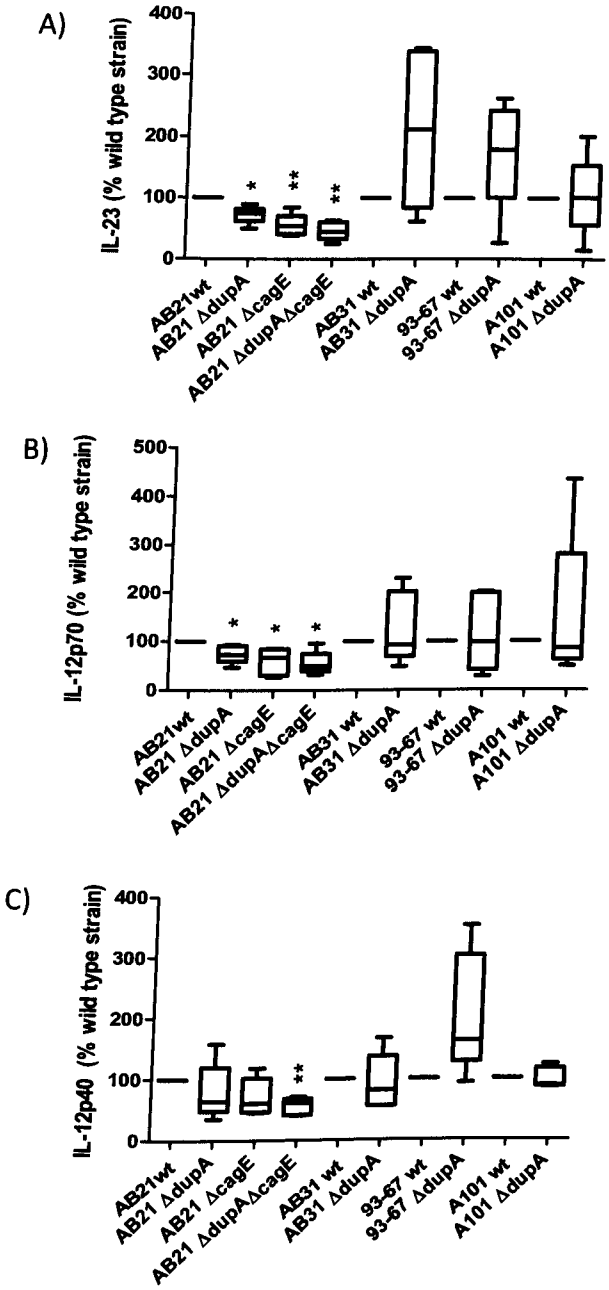


Figure 3.9 Effect of *dupA* and *cagE* mutations on secretion of IL-12 family cytokines by *Hp*-stimulated MoDCs. MoDCs from 6 donors were incubated with *Hp* wild-type strains AB21, AB31, 93-67 and A101 and isogenic *dupA* and *cagE* mutants for 24 hours. Cytokine levels in 24 hour supernatants were measured by ELISA. Bars represent mean of duplicate ELISAs on supernatants from 3 wells, error bars depict standard deviations. Strains AB21 and AB31 have an active *dupA* type, but strains 93-67 and A101 do not. Results are

expressed as a percentage of the wild-type parental strain. A) IL-12p70, B) IL-23, C) p40. * denotes $p<0.05$, ** $p<0.005$.

Mutation of *dupA* reduced IL-12p70 (1.4-fold, p=0.015) and IL-23 (1.4-fold, p=0.039) secretion by AB21 *Hp*-stimulated MoDCs, but mutation of *cagE* had a still greater effect reducing IL-12p70 1.5-fold, p=0.013 and IL-23 1.9-fold, p=0.0016. IL-12p40 levels were only significantly reduced in the double mutant in the AB21 *Hp* strain background compared to wild-type (1.6 -fold, p=0.0023). Mutation of *dupA* in the AB31 strain background had no significant effect on IL-12 family cytokine levels but variation was wide (Figure 3.9).

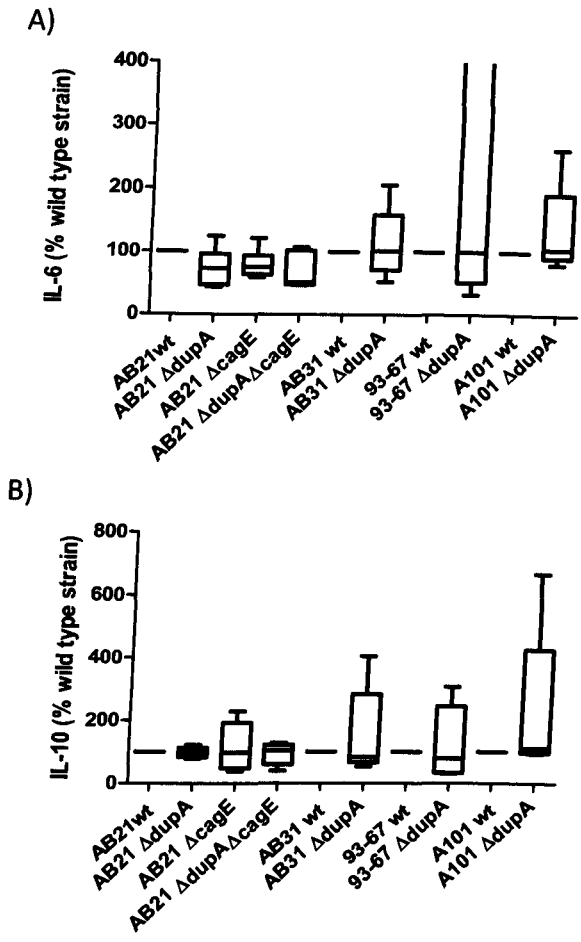


Figure 3.10 Effect of *dupA* and *cagE* mutations on secretion of IL-6 and IL-10 by *Hp*-stimulated MoDCs. MoDCs from 6 donors were incubated with *Hp* wild type strains AB21, AB31, 93-67 and A101 and isogenic *dupA* and *cagE* mutants for 24 hours. Cytokine levels in 24 hour supernatants were measured by ELISA. Bars represent mean of duplicate ELISAs on supernatants from 3 wells, error bars depict standard deviations. Strains AB21 and AB31 have an active *dupA* type, but strains 93-67 and A101 do not. Results are expressed as a percentage of the wild-type parental strain. A) IL-6, B) IL-10.

There were trends towards reduced IL-6 in the AB21 *Hp dupA* mutant, *cagE* mutant and double mutant, but these did not reach statistical significance.

Mutation of *dupA* and *cagE* did not have any effect on IL-10 secretion by *Hp*-stimulated MoDCs.

Relative cytokine levels were compared using this data set of cytokine measurements in the supernatants of DCs from 6 different donors co-cultured for 24 hours with AB21 and AB31 *Hp* wild-type strains. IL-12p40 was found at the highest concentration followed by IL-12p70 (3.5-fold and 2.1-fold lower with AB21 and AB31 *Hp* strains respectively), IL-8, IL-6, IL-23, IL-10 and finally IL-1β. Cytokines concentrations are listed in order of prevalence in Table 3.3 with their fold difference compared to IL-12p40 in brackets. IL-23 was 11.8-fold and 14.6-fold lower than IL-12p70 in the AB21 and AB31 *Hp* co-culture supernatants respectively.

	AB21 wt	AB31 wt
	Median cytokine level in pg/ml (fold difference less than p40)	
IL-12p40	431746	206401
IL-12p70	124564 (3.5)	100668 (2.1)
IL-8	91115 (4.7)	57400 (3.6)
IL-6	27588 (16)	13471 (15)
IL-23	10552 (41)	6900 (30)
IL-10	1306 (331)	1017 (203)
IL-1β	1072 (403)	702 (294)

Table 3.3 Relative cytokine levels. MoDCs from 6 donors were incubated with *Hp* AB21 and AB31 wild-type strains for 24 hours. Cytokine levels in 24 hour supernatants were measured by ELISA. Mean cytokine concentrations for each donor were calculated based on duplicate ELISAs on supernatants from 3 wells. The median of these values for the 6 donors has been included in the table, with fold difference less than IL-12p40 shown in brackets.

3.3.3 Effects of *Hp*-Stimulated DC Supernatants on the Differentiation of Naïve T Cells

To determine how the *Hp*-stimulated MoDCs influenced naive T cell differentiation supernatants from MoDCs co-cultured with *Hp* were cultured with naive T cells for 5 days and IFNγ and IL-17 were measured in the resulting supernatants. Naive T cells were isolated from PBMCs by $CD4^+$ followed by $CD45RA^+$ positive selection using magnetic beads, as described in section 2.1.1. The DC supernatants were filtered to remove any residual bacterial components. As no antigen presenting cells were present in this system anti-CD3/CD28 beads were used to provide the appropriate signals to the T cells. Wells without T cells and anti-CD3/CD28 were included for each condition to exclude the possibility that any IFNγ or IL-17 was being produced by the DCs themselves in response to the *Hp*. All of these wells had cytokine levels below the limit of sensitivity of the ELISAs used.

The *Hp*-stimulated DC supernatants provoked a very strong IFNγ response (Figure 3.11A) but IL-17 T cell secretion was only just above background levels (Figure 3.11B). High concentrations of IL-12, IFNγ and Th1 cells could have inhibited Th17 differentiation and IL-17 secretion (see section 3.4.3 in discussion).

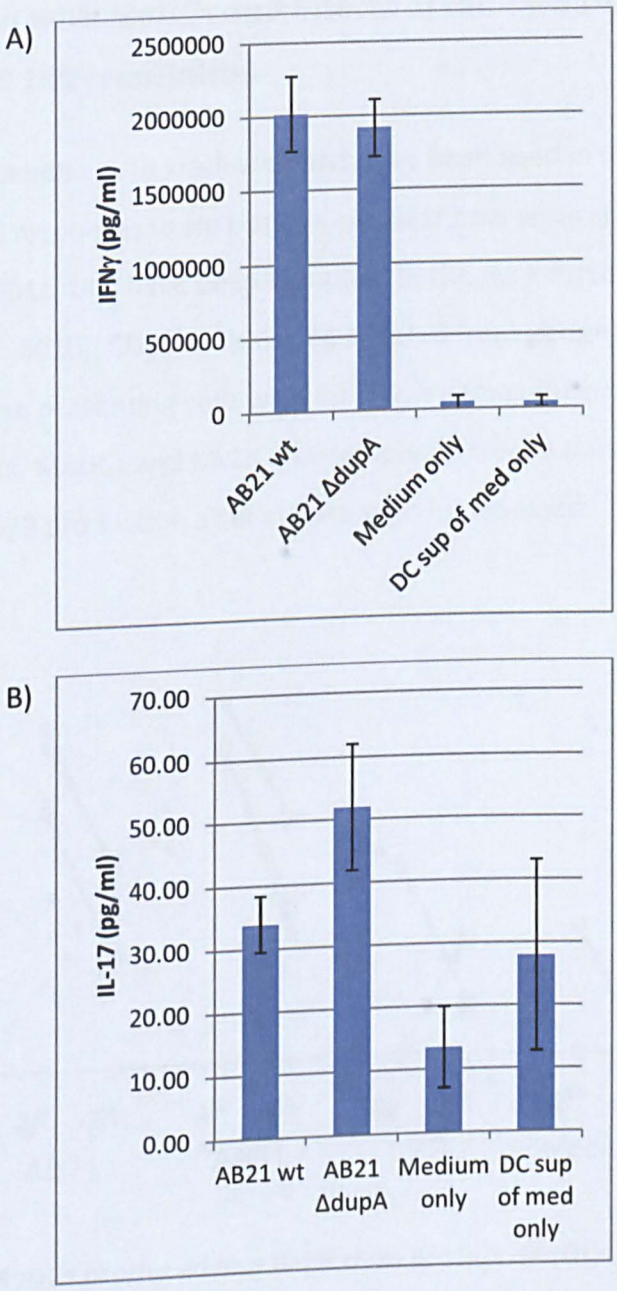


Figure 3.11 IFNγ and IL-17 responses upon adding supernatants from *Hp*-stimulated MoDCs to naive T cells. Supernatants from *Hp*-stimulated MoDCs and anti-CD3/CD28 beads were added to naive T cells (89% $CD4^+CD45RA^+$) and cultured for 5 days. IFNγ and IL-17 in the supernatants was measured by ELISA. Bars represent mean concentrations of 4 wells measured in duplicate. Error bars represent standard deviations.

3.3.4 Stimulation of CD1c+ Myeloid DCs (MyDCs) with *Hp*: Comparison with MoDCs and Effects of the Two DC Subtypes on Naive T Cell Differentiation

MoDCs are convenient to work with and have been used in the majority of studies on DC responses to *Hp* but it is not clear how accurately they reflect gastric DCs. $CD1c^+$ DCs have been identified in the *Hp*-infected gastric mucosa (Khamri et al., 2010). $CD1c^+$ cells can be isolated from peripheral blood and used as antigen presenting cells without any *in vitro* culture with cytokines or growth factors. MoDCs and MyDCs were isolated from 3 donors and their IL-23 and IL-12p70 production after culture with *Hp* assessed.

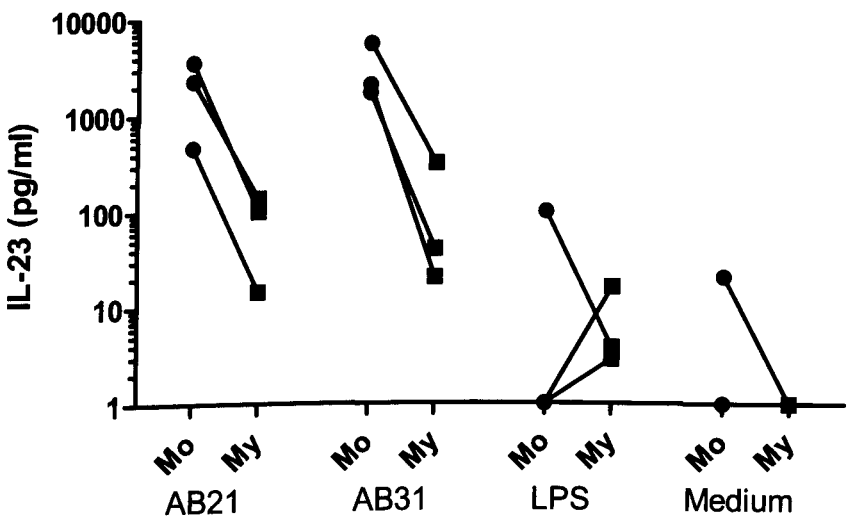


Figure 3.12 MyDCs produced less IL-23 than MoDCs. MyDCs isolated by positive selection from peripheral blood of 3 donors (typically >95% $CD1c^+$) and MoDCs from the same donors were cultured with *Hp* for 24 hours. IL-23 levels in the supernatants were determined by ELISA. Each point represents the mean IL-23 concentration for a single donor, calculated from duplicate ELISAs performed on supernatants from 3 wells.

IL-23 levels were lower in the supernatants of MyDCs incubated with wild-type *Hp* strains than MoDCs incubated with the same bacteria but the differences did not reach significance (median IL-23 levels in AB21 *Hp* strain MyDC supernatant 25pg/ml vs 2321pg/ml in MoDC supernatant, p=0.25; 101pg/ml in MyDC supernatant vs 2146pg/ml in MoDC supernatant for AB31 *Hp* strain, p=0.25 using Wilcoxon matched-pairs signed rank test).

No reduction in IL-23 was seen with the *dupA* or *cagE* mutants in the MyDC supernatants of these 3 donors (data not shown). IL-23 concentrations were lower with LPS than with *Hp* stimulation, as noted previously. In most cases IL-23 was undetectable when the DCs were cultured with medium only but low levels were detected in the MoDC supernatant of one donor.

An IL-12p70 ELISA with limit of sensitivity 4.0pg/ml was unable to detect cytokine in MyDC supernatants from any of the 3 donors. The MoDC supernatants in contrast contained median IL-12p70 levels of 19072pg/ml and 41932pg/ml following stimulation with *Hp* AB21 and AB31 strains respectively.

Having shown profound differences in the responses of MoDCs and MyDCs to *Hp*, the impact of this on naive T cell differentiation was examined. MoDCs and MyDCs from 3 donors that had been co-cultured with *Hp* for 24 hours were cultured with autologous naive T cells for 5 days and IFNγ and IL-17 levels in the supernatants were measured by ELISA.

The very low IL-12p70 concentrations in supernatants of MyDC co-cultured with *Hp* led to the hypothesis that co-culture of *Hp*-primed MyDCs with naive T cells would result in reduced Th1 cell differentiation and much lower IFNγ concentrations.

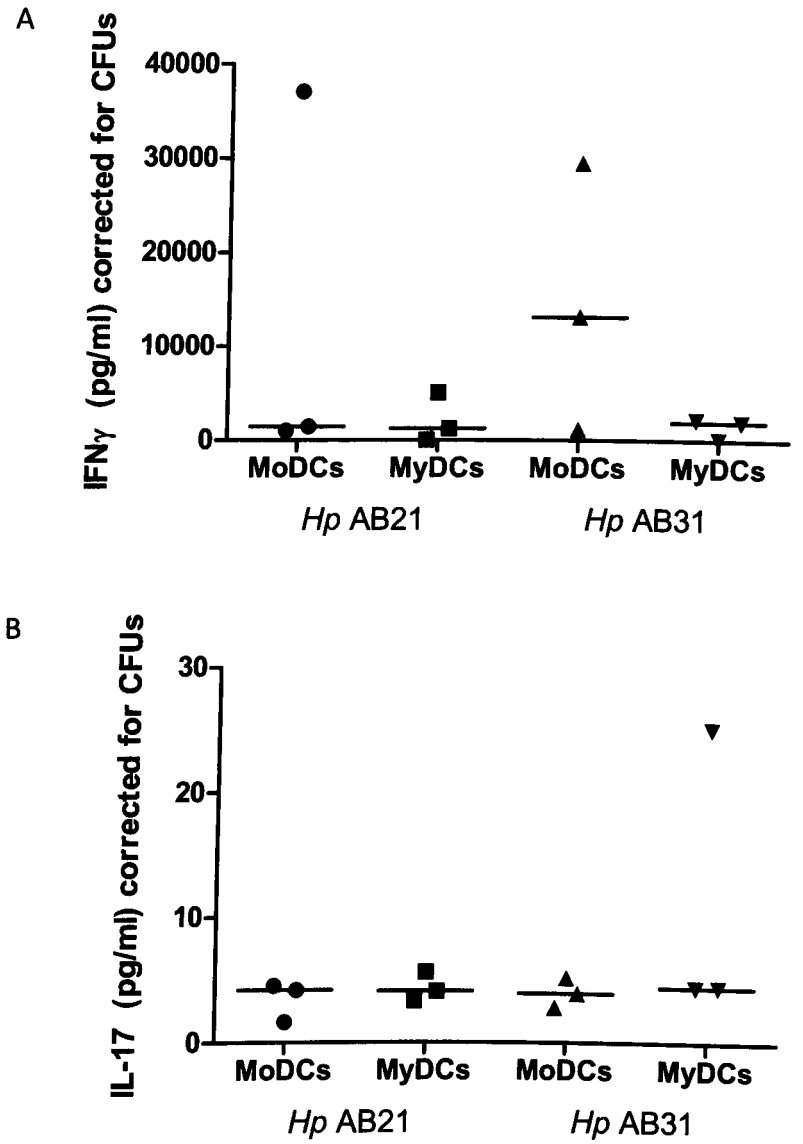


Figure 3.13 Relative cytokine levels produced by naive T cells incubated with *Hp*-stimulated MoDCs and MyDCs for 5 days. A) IFNγ, B) IL-17. MoDCs and MyDCs from 3 donors that had been exposed to *Hp* for 24 hours were cultured with autologous naive T cells (95% $CD4^+CD45RA^+$) for 5 days and supernatants collected. IFNγ and IL-17 levels were tested by ELISA. Points represent the mean cytokine concentration measured in supernatants from a single well in duplicate for each donor. Bars denote medians of the 3 donors.

MyDC and T cell numbers were limiting, so the DCs stimulated with each *Hp* strain were only co-cultured with T cells in a single well for each donor.

MoDCs tended to produce more IFNγ than MyDCs, in keeping with their strong IL-12 response, but variation amongst the 3 donor samples was wide and the differences did not reach statistical significance (Figure 3.13A). However co-culture of *Hp*-stimulated MyDCs with naive T cells did result in substantial IFNγ production, despite the lack of detectable IL-12p70 secreted by the *Hp*-stimulated MyDCs.

Low levels of IL-17 were produced by both types of DC. The median IL-17 levels for Mo- and MyDCs were very similar. T cells from one of the 3 donors cultured with AB31 *Hp*-stimulated MyDC had a strong IL-17 response. (Figure 3.13B).

IFNγ levels were higher than IL-17 levels for T cells stimulated by both types of *Hp*-primed DCs but the differences were not statistically significant. The difference was more marked in the MoDCs due to the strong IFNγ response to AB31 *Hp*-stimulated MoDC supernatants.

No consistent reduction in IFNγ or IL-17 was found with T cells that had been stimulated by DCs previously exposed to *Hp* strains with mutated *dupA* or *cagE* (data not shown).

Thank you to Qunwei Wang and Dr Hester Franks, Academic Unit of Clinical Oncology, University of Nottingham for their help with DC preparation and to Dr Chistian Marx, Helle Skjoldmose and Benoit Biousse who performed some of the cytokine ELISAs from which data are presented in this chapter.

3.4 DISCUSSION

3.4.1 Optimization of Conditions for Co-Culture of Monocyte-Derived DCs with *Hp*

The MoDC model was used initially as it is well recognized and has been the main published strategy used to study the human DC response to *Hp*.

IL-23 levels were lower at 48 hours compared to 24 hours (Figure 3.2) in line with Khamri *et al.*'s findings that *Hp*-induced IL-23 secretion peaked at 24 hours (Khamri et al., 2010). There was no significant increase in IL-12p70, p40 or IL-10 at 48 hours compared to 24 hours either (data not shown). 24 hours was therefore selected as an appropriate duration for which to incubate *Hp* with the DCs for subsequent experiments.

The dose-dependent production of cytokines by MoDCs in response to *Hp* was in keeping with the findings of others (Kranzer et al., 2004, Bimczok et al.). Cytokine production increased up to the maximum MOI of 50, however, an MOI of 10 was selected for subsequent experiments to provide a more physiologically relevant dose. Activation markers did not increase further with higher MOIs (Figure 3.4 and Table 3.2). Cell death has been reported at MOIs greater than 10 (Kranzer et al., 2004). This MOI is thought to approximate levels of infection *in vivo* and has been used by a number of authors in similar studies (Khamri et al., 2010, Kranzer et al., 2004, Kranzer et al., 2005, Guiney et al., 2003).

Hp 26695 was included in the initial optimization experiments (Figures 3.2 and 3.3), for comparison, as it was the strain used in a similar study by Khamri *et al.* It stimulated similar levels of cytokine secretion to the AB31 strain (Figures 3.2 and 3.3). Kranzer *et al.* measured levels of 6 different cytokines released by MoDCs from one donor stimulated by 8 different strains of *Hp* and found little strain to strain variation (Kranzer et al., 2005). However, others have reported differences in activation marker expression and

cytokine production in response to different strains (Hansson et al., 2006, Andres et al., 2011). The levels of cytokines produced by *Hp*-stimulated DCs vary widely between different studies. This is likely to be due to a combination of factors including different *Hp* strains and MOIs, different methods for isolating $CD14^+$ cells, maturing them into DCs and concentrations for plating them and different time points. The purity of recombinant IL-4 and GM-CSF used may also be critical and can vary from batch to batch.

The relative levels of IL-12 family members secreted by *Hp*-stimulated MoDCs are depicted in Figure 3.3. Concentrations of IL-12p40, IL-12p70 and IL-23 but not IL-12p35 were measured. Others have taken a similar approach (Fehlings et al., 2012, Hussein et al., 2010). However IL-12p35 ELISAs are commercially available so direct measurement of IL-12p35 could be included in future studies. The p40 subunit was by far the most prevalent, to the extent that more of this was secreted than IL-12p70 and IL-23 combined. This is in keeping with previous findings that IL-12p40 is found in excess of p70 when monocytes are stimulated with *E.coli* LPS (Snijders et al., 1996). Whereas IL-12p35 is only secreted as part of IL-12p70, IL-12p40 can be secreted alone (Snijders et al., 1996). The excess IL-12p40 may be present as monomers, homodimers, or with a subunit which has not yet been described.

The role of IL-12p40 monomers and homodimers is not entirely clear. Levels of both IL-12p40 monomer and homodimer increased in the serum of C57BL/6 mice after injection of salmonella LPS. Mice treated with p40 homodimer had reduced IFNγ responses to endotoxin (Heinzel et al., 1997), suggesting an inhibitory or blocking role for the homodimer. Studies using human cell lines also supported an antagonist role for IL-12p40 homodimers, which blocked IL-12 binding *in vitro* (Ling et al., 1995). Homodimers of IL-12p40 may have different effects on different cell populations as they were reported to reduce IFNγ production by $CD4^+$ splenocytes but increase IFNγ production from the $CD8^+$ splenocytes (Piccotti et al., 1997). IL-12p40 levels are increased in the CNS of patients with multiple sclerosis (Fassbender et al., 1998) and mice with EAE (Bright et al., 1998). The IL-12p40 homodimer has

proinflammatory effects on microglia and macrophages including increasing induction of nitric oxide synthase (Pahan et al., 2001, Jana et al., 2009), TNFα (Jana et al., 2003) and lymphotoxin-α (Jana and Pahan, 2009) and increases NF-κB activation in the BV-2 cell line (Pahan et al., 2001). The IL-12p40 homodimer also has a role in macrophage chemotaxis (Russell et al., 2003).

Becker *et al.* studied *IL12B* (p40 gene) expression in various tissues using transgenic mice expressing firefly luciferase under control of the *IL12B* promoter and found constitutive *IL12B* expression in $CD11c^+$ lamina propria DCs in the terminal ileum (Becker et al., 2003). Unfortunately stomach was not included in their panel of tissues.

The predominance of IL-12p70 over IL-23 suggests that *Hp*-stimulated DCs are likely to be pro-Th1 rather than pro-Th17. However, these findings are discrepant with the relative cytokine levels found in snap frozen human gastric biopsies (Figure 5.10). This might suggest that cells other than DCs present in the gastric mucosa produce IL-23, or that the *in vitro* MoDC model used here does not reflect gastric DCs (discussed further in chapter 5).

Phenotyping of *Hp*-stimulated MoDCs demonstrated <0.5% $CD14^+$ and >90% $HLA\text{-}DR^+$ and $CD11c^+$, consistent with a mature MoDC cell type. The activation markers CD80, CD83 and CD86 were present on >80% *Hp*-stimulated cells after 24 hours, confirming *Hp* as a strong inducer of DC activation/maturation. The percentage of $CD11c^+$ cells in Table 3.2 do not increase with *Hp* stimulation, but this is misleading as the Mean Fluorescence Intensity (MFI) does increase but the cut-off has been chosen based on the two populations in the unstimulated sample (Figure 3.5).

Other authors have also noted increased activation of human MoDCs following stimulation with *Hp in vitro*. In keeping with the findings here Andres *et al.* found increased CD80 and CD86 after 24 hours of stimulation with *Hp* MOI 10 (Andres et al., 2011), but most investigators have used longer incubations with *Hp*. Kranzer *et al.* found increased CD80, CD83, CD86 and HLA-DR expression on DCs at 72 hours (Kranzer et al., 2004, Kranzer et al.,

2005). Hansson *et al.* and Hafsi *et al.* found increased HLA-DR, CD80 and CD86 and Hafsi *et al.* also reported increased CD83 after 48 hours (Hansson et al., 2006, Hafsi et al., 2004). Mitchell *et al.* also noted increased CD40 and CCR7 at 48 hours upon stimulation of MoDCs with paraformaldehyde fixed *Hp* (Mitchell et al., 2007). Some authors have reported MFIs rather than percentages of cells positive for activation markers (Kranzer et al., 2004, Mitchell et al., 2007) but Bimczok *et al.*'s findings on DCs from human gastric tissue were similar to our own with HLA-DR^{+}CD11c^{+} MoDCs having >80% CD80, CD83 and CD86 after *Hp* stimulation, though they found very high CD86 expression in their unstimulated controls (Bimczok et al.). Studies using mouse BMDCs have noted similar upregulation of CD80, CD86 in response to *Hp* stimulation (Rad et al., 2007).

As mentioned above, there is controversy as to whether *Hp* increases DC activation *in vivo.* Bimczok *et al.* found increased CD11c, CD83 and CD86 on gastric DCs from infected patients compared to uninfected controls, although the percentages of these markers were much lower than those found on MoDCs stimulated with *Hp in vitro* in the same study (Bimczok et al.). Oertli *et al.* found little or no CD80, CD83 or CD86 on gastric DCs with no difference between the infected and uninfected groups. They argue that this may lead to tolerance and facilitate persistence of the infection but unfortunately no mechanistic experiments were carried out (Oertli et al., 2012). Both studies were small with only 3-4 patients each in the infected and uninfected group.

3.4.2 Effects of Mutating Virulence Factor Genes *dupA* and *cagE* on Cytokine Secretion by *Hp*-Stimulated MoDCs

Guiney *et al.* found reduced IL-12p70 secretion by MoDCs stimulated by *Hp* with mutated *cagE* (Guiney et al., 2003). Galgani *et al.* were in agreement that IL-12 secretion by MoDCs was reduced with an isogenic *cagE* mutant and also found reduced levels of IL-1 and TNFα (Galgani et al., 2004). Khamri *et al.* found reduced IL-23 and a trend towards reduced IL-1β secretion from MoDCs stimulated with the isogenic *cagE* mutant and reduced IL-17 secretion

by autologous $CD4^+$ T cells stimulated co-cultured with DCs primed with the isogenic *cagE* mutant compared to the wild-type *Hp* strain (Khamri et al., 2010). However, Kranzer *et al.* did not find any effect of *cag* or *vacA* status on MoDC cytokine secretion or activation marker expression with their panel of 8 different wild type strains of *Hp* with a range of different virulence factor genotypes (Kranzer et al., 2005). Andres *et al.* did not find any significant difference in IL-12p70, TNF-α, IL-6 or IL-1β secretion by MoDCs stimulated with 20 different clinical isolates of *Hp* analyzed according to *cagA*, *cag*PAI, *vacA* or *babA* status, though trends were observed for IL-12p70 and *cag*PAI (Andres et al., 2011). Recent studies by Oertli *et al.* using *Hp* strains deficient in γ-glutamyl transpeptidase and VacA expression to infect murine BMDCs *in vitro* and mice *in vivo* suggest that these virulence factors contribute to generating tolerogenic DCs that promote Treg generation (Oertli et al., 2013). No data has yet been published on the effect of mutating *dupA* on the DC response to *Hp*.

Production of a panel of 8 different cytokines in response to *Hp* strain AB21 and its isogenic *dupA*, *cagE* and double mutants by MoDCs were initially studied in a single donor. The AB21 strain was used for this work as the full panel of isogenic mutants was available. Cytokine secretion was reduced in both the *dupA* and *cagE* mutants for IL-12p70, IL-23, p40, IL-6 and IL-10, but the effects were more marked with mutation of *cagE*. For IL-1β only *cagE* mutation had a significant effect on cytokine levels. Neither mutation reduced levels of IL-8 secreted by MoDCs, though strains with the *cag*PAI are known to cause increased IL-8 secretion by gastric epithelial cells via NF-κB (Sharma et al., 1998, Yamaoka et al., 1998). To confirm these results MoDCs from 5 further donors were tested and the panel of *Hp* strains expanded to include another strain with the more active form of *dupA*, *dupA1* (AB31) and 2 strains with the less active *dupA2* (93-67 and A101). Wide variation was seen between absolute levels of cytokines produced by individual donors (Figures 3.9 and 3.10), as has been noted by other authors (Kranzer et al., 2004, Andres et al., 2011). IL-12p70 and IL-23 secretion was reduced with all the

AB21 mutants, though the *cagE* mutant had a more marked effect than the *dupA* mutant, but mutation of *dupA* on the AB31 background did not have any effect on levels of cytokines secreted by the *Hp*-stimulated MoDCs (Figure 3.9). This raises the question whether this is an AB21 strain-specific effect or whether the same phenomenon will occur in other *Hp* strains with active *dupA* secretion systems. However subsequent work has suggested that the "AB31 *dupA* mutant" may not be a true isogenic mutant. The effect of mutating *dupA* in the AB31 strain should be reassessed with recently sequenced and confirmed strains. No reductions in cytokine secretion with mutation of *dupA* were seen in strains *Hp* 93-67 or *Hp* A101, which was expected as these strains do not have a functional *dupA* genotype (Hussein et al., 2010).

When results of the 6 donors were combined, mutation of *dupA* and/or *cagE* no longer had any significant effect on the levels of IL-6 and IL-10 produced in response to *Hp.* This highlights the technical difficulties of these experiments and the importance of ensuring that results are reproducible using cells from multiple donors.

The *Hp* strains were grown on DENT *Hp*-selective plates (which contain trimethoprim, vancomycin, cefsulodin and amphotericin B) to minimize the chance of contamination and taken out of the freezer a few days before the experiment to try to encourage consistent growth and viability. *Hp* has modified PAMPs which are less potent stimuli than those of many other bacteria (Lee and Josenhans, 2005, Gewirtz et al., 2004) and is known to stimulate less cytokine production from MoDCs than *Salmonella enterica* and *E. coli* (Guiney et al., 2003, Hafsi et al., 2004) and less cytokine production from murine BMDCs than *Acinetobacter lwoffi* or *E. coli* (Kao et al., 2006b). It is therefore likely that small numbers of contaminating bacteria could have a highly significant effect on the total cytokine levels in a well.

The amount of *Hp* required for a particular MOI was calculated using the optical density method, which is widely used to determine bacterial

concentration (Kranzer et al., 2005, Mitchell et al., 2007, Khamri et al., 2010, Bimczok et al., 2010). The *Hp* preparation was also plated out at a range of dilutions so that the actual number of CFUs could be determined. Results were adjusted for CFUs in order to allow fair comparison between different strains and mutants.

Mutation of *dupA* appears to have a more marked effect on cytokine secretion by *Hp*-stimulated monocytes than on cytokine secretion by *Hp*-stimulated DCs *in vitro* (Hussein et al., 2010). This may be at least partly due to different expression of PRRs, such as TLRs by these different cell types. Monocytes express higher levels of TLR 2, 4, 5 and 8 mRNA than $CD11c^+$ immature DCs (Kadowaki et al., 2001). In keeping with these findings differentiation of monocytes into DCs leads to loss of their ability to secrete IL-1β in response to TLR2 ligand (Pam3Cys), LPS and *S. epidermidis* (Netea et al., 2009). However, monocytes are more susceptible to *Hp*-induced apoptosis than DCs (Galgani et al., 2004). Macrophages, unlike monocytes, are present in the *Hp* infected stomach (Quiding-Jarbrink et al., 2010). Monocyte-derived macrophages have similar TLR2 and 5 expression and higher TLR4 expression compared to monocytes (O'Mahony et al., 2008). It is possible that they could be the *dupA* responsive link *in vivo.*

Other cytokines may be relevant in addition to the the cytokines measured here. TGF-β is important for both Th17 and Treg differentiation (Bettelli et al., 2006) and three isoforms are expressed in mammals: TGF-β1, TGF-β2 and TGF-β3 (Massague, 1990, Bandyopadhyay and Raghavan, 2009, Ribeiro et al., 1999). However TGF-β is synthesized and secreted in a pro-form in which it is covalently associated with latency associated peptide (LAP) (Massague, 1990, Bandyopadhyay and Raghavan, 2009), therefore though TGF-β is very widely expressed at the mRNA level this may not reflect its biological activity. Activated circulating TGF-β has a very short half-life (Massague, 1990). Acid activation is used to release active TGF-β from its latent complex when TGF-β is measured by ELISA but whether this reflect levels biological activity *in vivo*

is unclear. Concentrations of LAP may provide a surrogate measure of TGF-β activity as release from the LAP-TGF-β complex is known to be a key point in regulation of TGF-β activity (Ribeiro et al., 1999). IL-18 is increased in human *Hp*-infected gastric mucosa (Yamauchi et al., 2008) and IL-18 from *Hp*-stimulated DCs promotes Treg differentiation (Oertli et al., 2012). IL-27 induces Tr1 Tregs but inhibits Th17 differentiation (Awasthi et al., 2007, Batten et al., 2006). Though this study has focussed on measurement of DC cytokines and activation markers gene expression profiling of murine BMDCs showed induction of a wide range of cytokines and proinflammatory genes following *Hp* exposure, including enzymes involved in prostaglandin and nitric oxide synthesis and a number of chemokines, suggesting that DCs may have a role in recruiting immune cells to the site of infection as well as in their differentiation (Rad et al., 2007).

3.4.3 Effects of *Hp*-Stimulated DC Supernatants on Naïve T Cell Differentiation

Adding DC supernatants to naive T cells avoids the need to bleed the donor twice to obtain the naive T cells after the $CD14^+$ cells have had 5-6 days to develop into immature MoDCs. Artificial CD3 and CD28 stimulation is required in the absence of antigen presenting cells. It was important to exclude IL-4 (used with GM-CSF to generate immature MoDCs from $CD14^+$ monocytes) from the medium when the DCs were co-cultured with *Hp* to avoid any skewing of T cell differentiation. There was a very strong IFNγ response from the T cells (necessitating 1 in 8000 dilution to quantitate it by ELISA), with a weak IL-17 response, only just above background (Figure 3.11). During early experiments characterizing Th17 as a separate lineage it was noted that IL-12 suppressed IL-17 production (Aggarwal et al., 2003, Hoeve et al., 2006, Annunziato et al., 2007). IFNγ was also shown to reduced Th17 differentiation and IL-17 secretion (Park et al., 2005, Harrington et al., 2005). More recently T-bet was found to inhibit Th17 differentiation via prevention of Runx1-

mediated RORγt activity leading to reduced IL-17 production in the absence of T-bet (Lazarevic et al., 2011).

The naive T cells used were purified by positive $CD4^+$ and $CD45RA^+$ selection but were only 89% pure, raising the possibility that a small fraction of contaminating cells (e.g. $CD8^+$ T cells, $CD4^+CD45RA^-$ or NK cells) could have contributed to the high levels of IFNγ. Purer naive T cell populations (95% $CD4^+CD45RA^+$) were obtained for later DC:T cell co-culture experiments (Figure 3.13).

Anti-CD3/CD28 T cell stimulating beads seemed to exaggerate the IFNγ response, when compared with the response following co-culture with *Hp*-stimulated DCs directly, but the IL-17 response was of similar magnitude with the two different methods (Figures 3.11 and 3.13). However these were separate experiments with cells from different donors so it is difficult to draw any firm conclusions.

Other authors have co-cultured autologous naive T cells of varying degrees of purity with *Hp*-pulsed MoDCs and measured IFNγ production. Hafsi *et al.* stimulated a more than 80% $CD4^+CD45RA^+$ T cell population with *Hp*-pulsed DCs and found IFNγ secretion, which was increased with a membrane preparation of *Hp* compared to cytosolic preparation or whole bacteria (Hafsi et al., 2004). There was an associated increase in *TBX21* (gene encoding T-bet) expression and reciprocal decrease in *GATA3* expression. NK cells incubated with *Hp* secreted larger amounts of IFNγ than the T cell population, though there was no increase in their cytotoxic activity (Hafsi et al., 2004). Mitchell *et al.* found naive T cells (negatively selected, purity not stated) incubated with *Hp*-primed DCs for 48 hours produced less IFNγ than those stimulated for 8 hours. This was in keeping with their findings of reduced IL-12 production by MoDCs at the longer time point (Mitchell et al., 2007). Bimczok *et al.* pulsed MoDCs and gastric $HLA\text{-}DR^+$ DCs with *Hp* for 2 hours, then cultured the DCs with autologous $CD4^+$ T cells (total, not naive) for 3 days. ELISAs on the

supernatants showed low levels of IL-10 and high levels of IFNγ, which were similar for the MoDCs and gastric DCs (Bimczok et al.).

Khamri *et al.* cultured *Hp*-stimulated MoDCs with autologous negatively selected naive $CD4^+$ T cells for 5 days and found increased $CD3^+IL\text{-}17^+$ cells compared to co-culture with unstimulated MoDCs (Khamri et al., 2010). $CD4^+IFN\gamma^+$ cells were also found after culture of the naive $CD4^+$ T cells with *Hp*-stimulated MoDCs but the relative levels of Th17 and Th1 responses were not compared at cellular or cytokine level (Khamri et al., 2010).

DeLyria *et al.* cultured *Hp*-pulsed antigen presenting cells from *Hp*-infected mice with spleen-derived $CD4^+$ T cells and found higher levels of IL-17 than IFNγ in the supernatants by ELISA (DeLyria et al., 2009).

3.4.4 Stimulation of Myeloid DCs (MyDCs) with *Hp*: Comparison with MoDCs and Effects of the Two DC Subtypes on Naive T Cell Differentiation

The $CD1c^+$ MyDCs produced low levels of IL-23 in response to *Hp* compared to MoDCs (Figure 3.12) and no IL-12p70 was could be detected. Bimczok *et al.* reported a lack of IL-12p70 response by human gastric tissue DCs stimulated with *Hp,* and a corresponding lack of *IL12A* and *IL12B* mRNA by RT-qPCR (Bimczok et al.). In the same study MoDCs did secrete IL-12p70 in response to *Hp.* This suggests that MyDCs may be a better model for gastric DCs than MoDCs. Murine BMDCs also failed to produce IL-12p70 in response to *Hp* in one study (Wang et al., 2010), though others have found that BMDCs do secrete IL-12p70 in response to *Hp*, although at lower levels than when stimulated with *E.coli* LPS (Rad et al., 2007) or *A. lwoffi* (Kao et al., 2006b).

Autologous naive T cells stimulated by *Hp*-primed MoDCs tended to produce more IFNγ than those stimulated by *Hp*-primed MyDCs, especially when the AB31 *Hp* strain was used (Figure 3.13A) but the differences were not statistically significant, probably due to low donor numbers. The median levels of IL-17 produced by T cells stimulated by the different types of DC were almost identical (Figure 3.13B). The T cells cultured with *Hp*-stimulated

MyDCs made a substantial IFNγ response despite the lack of IL-12p70 production by the MyDCs. $CD4^+$ T cells cultured with *Hp*-stimulated gastric DCs (which did not produce IL-12p70) also produced a large IFNγ response (Bimczok et al.). In this paper total rather than naive $CD4^+$ T cells were used, so it is likely some already Th1-differentiated $CD4^+$ T cells were present. The authors postulate a role for DC-derived macrophage migration inhibitory factor (MIF) in Th1 differentiation (Bimczok et al.). MIF expression is increased in the gastric epithelium, T cells and macrophages in *Hp* infection and may have a role in the development of gastric cancer (He et al., 2006). MIF knockout mice had reduced IFNγ, reduced *T-bet* expression and reduced gastritis at 8 weeks (Wong et al., 2009). However, MIF secretion from MoDCs was reported to decrease following *Hp* stimulation (Fehlings et al., 2012).

IL-23 levels measured in the gastric mucosa using Luminex were much higher than IL-12p70 levels (Chapter 5 section 5.3.4). Whole gastric biopsies are homogenized for this method so this suggests that there may be another source of IL-23 within the gastric mucosa.

Other antigen presenting cells may also have a role. Macrophages are broadly divided into M1 macrophages, which are proinflammatory and microbicidal and M2 macrophages which have a role in resolution of inflammation and secrete IL-10 (Benoit et al., 2008). Markers of both M1 and M2 macrophages are increased in the gastric mucosa of *Hp*-infected patients (Quiding-Jarbrink et al., 2010).

Fehlings *et al.* compared the response of human blood-derived monocytes, MoDCs and macrophages to *Hp*. Surprisingly they did not detect any IL-12p70 secretion by monocytes in response to *Hp*, though they did find IL-12p40 and IL-23. IL-12p70 was secreted by MoDCs, but at lower levels than IL-23 (Fehlings et al., 2012) - opposite to my findings. This may reflect differences in the protocols used, for example Fehlings *et al.* pulsed the antigen presenting cells for only an hour whereas in this study *Hp* was co-cultured with DCs for 24 hours. Levels of IL-12p40 secreted by monocytes were approximately 100-

fold lower than those secreted by MoDCs, suggesting monocytes are not a major source of IL-12 family cytokines (Fehlings et al., 2012, Hussein et al., 2010). M1 macrophages produced pro-inflammatory cytokines including IL-12p40, IL-23, IL-12p70 (just detectable), IL-1β, IL-6 and low levels of IL-10. M2 macrophages produced IL-10 and low levels of proinflammatory cytokines, as expected (Fehlings et al., 2012).

DeLyria *et al.* cultured *Hp*-pulsed bone marrow-derived antigen presenting cells from *Hp*-infected mice with $CD4^+$ T cells and found that both IL-17 and IFNγ production were approximately halved if macrophages were used compared to DCs (DeLyria et al., 2009). Zhuang *et al.* also showed upregulation of *T-bet* and *RORγt* in murine splenic $CD4^+$ T cells with increased IFNγ and IL-17 secretion following incubation with *Hp*-infected macrophages (Zhuang et al.).

Other cells present in the stomach, but not present in our *in vitro* model, such as epithelial cells, may have important effects on DCs and T cell differentiation. Thymic Stromal Lymphopoietin (TSLP) secreted by epithelial cells can inhibit IL-12 secretion and promote Th2 responses (Rimoldi et al., 2005). Gastric epithelial cells secrete increased levels of TSLP, the chemokine CCL20 (MIP3α) and B cell activating factors (BAFF, also known as BLyS) upon exposure to *Hp* (Kido et al., 2010). Somatostatin, a peptide hormone secreted in the stomach, inhibited *Hp*-induced IL-12 secretion when added to BMDCs (Kao et al., 2006a). COX-2 and prostaglandin E_2 are upregulated by *Hp* in human PBMCs and gastric biopsies (Meyer et al., 2003, Pellicanò et al., 2007). Exogenous prostaglandin E_2 suppressed PBMC IL-12 and IFNγ responses and inhibition of COX-2 increased T-bet, IL-12 and IFNγ protein (Meyer et al., 2003, Pellicanò et al., 2007). Bimczok *et al.* showed that gastric stromal factors could suppress DC-driven Th1 differentiation but this did not appear to be due to TGFβ, prostaglandin E_2, IL-10 or TSLP (Bimczok et al., 2011).

In addition to its homing, imprinting and class-switching effects on DCs described in the introduction, the vitamin A metabolite retinoic acid favours

Treg differentiation at the expense of Th17 differentiation (Mucida et al., 2007). Balance of Treg/Th17 differentiation may also be affected by availability of AhR ligands (Quintana et al., 2008). Endogenous ATP release by monocytes enables them to secrete IL-1β in response to TLR2 or TLR4 whereas macrophages needed additional exogenous ATP to do so (Netea et al., 2009). ATP produced by commensal bacteria can drive Th17 differentiation in the lamina propria (Atarashi et al., 2008). Commensal bacteria may promote peripheral Treg development and shape the repertoire of Tregs in the gut as many colonic Tregs had T cell receptor specificities for antigens of commensal bacteria that were not found in other tissues (Lathrop et al., 2011). Co-infection models have shown that *Hp* infection can modulate the immune response to intestinal infection and vice versa (Fox et al., 2000).

In addition to differentiation of naive $CD4^+$ T cells the gastric environment may also alter the phenotype of T helper cells recruited to the stomach, as T helper cells have some plasticity. For example mouse and human Tregs can be induced to become IL-17 producers in proinflammatory environments *in vitro* (Xu et al., 2007, Koenen et al., 2008, Nyirenda et al., 2011) and Th17 cells can take on Th1 characteristics *in vivo* (Bending et al., 2009, Shi et al., 2008).

All this complexity makes it difficult to model DCs in the human stomach. Mouse models have the advantages of a complete *in vivo* system with multiple cell types present in the stomach. However there are differences in T cell differentiation between mice and humans (reviewed in (Laurence and O'Shea, 2007)) and even the commensal bacteria present may affect these processes, which may cause differences in mice from different animal houses, as well as between mice and humans. Gastric DCs are the ideal choice as the basis of an *in vitro* human model system, but are difficult to obtain as large surgical resections of gastric tissue are needed to extract sufficient numbers of cells. The results presented here suggest that $CD1c^+$ MyDCs may be a better model of gastric DCs than the more commonly used MoDCs. $CD1c^+$ DCs can be isolated from blood. Larger volumes of blood are required than for

extraction of $CD14^+$ cells for development of MoDCs but once extracted the $CD1c^+$ DCs are ready to use.

In this chapter human DCs produced IL-23 in response to *Hp,* with maximal responses when *dupA*$^+$ and *cagE*$^+$ strains were used, and this in turn led to Th17 differentiation. Next PBMCs from *Hp*-infected patients and uninfected controls were stimulated with *Hp* or control antigen (tetanus) to assess whether *Hp* infection results in a systemic *Hp*-specific Th17 response *in vivo,* as described in the next chapter.

CHAPTER 4

PERIPHERAL BLOOD TH17, TH1 AND TREG RESPONSES TO *HP*

4. PERIPHERAL BLOOD TH17, TH1 AND TREG RESPONSES TO *HP*

4.1 INTRODUCTION

Though the site of *Hp* infection is the stomach, changes have also been demonstrated in the peripheral blood of *Hp*-infected patients. The most obvious of these is the systemic antibody response, both IgA and IgG, to *Hp* which is in widespread clinical use as a marker of current or previous infection. As peripheral blood is relatively easy to sample any disease-associated or prognostic marker found here would be an attractive candidate to develop for clinical use. Although the anti-*Hp* antibody response is well characterized, it is unclear if it has any effect on Hp colonization density (Robinson et al., 2007). However, in this chapter I will focus on the less well characterized Th17, Th1 and Treg cells and their associated cytokines in the peripheral blood.

4.1.1 PBMC Proliferative Responses to *Hp*

Early investigators found that PBMCs from both individuals who were seropositive for *Hp* and those with negative *Hp* serology proliferated in response to *Hp* antigen, indeed several groups noted that proliferation in the *Hp*+ group was reduced compared the *Hp*- group (Karttunen et al., 1990, Birkholz et al., 1993, Fan et al., 1994). The proliferative response to *Hp* in those seronegative to *Hp* suggests that either the seronegative subjects have previously been exposed to *Hp* and the cellular memory response outlasts the antibody response, or that there is cross-reactivity between epitopes, such as PAMPs recognized on *Hp* and other microorganisms. The theory that the immune responding cells are not restricted to a small number of *Hp*-specific memory clones is supported by evidence from Di Tommaso *et al.* who cloned *Hp*-specific $CD4^+$ T cells from the peripheral blood and found them to be polyclonal in terms of both TCR usage and MHC restriction (Di Tommaso et al., 1995). Furthermore Quiding-Jarbrink *et al.* found that the majority of $IFN\gamma^+$ cells following *Hp* stimulation were $CD45RA^+$ in both *Hp*+ and *Hp*- subjects, implying that these responder cells were naïve and had not

previously been stimulated (Quiding-Jarbrink et al., 2001b). Malfitano *et al.* also found evidence of *Hp* stimulating naive T cells including a lower CD45RO/RA ratio of response to *Hp* antigen than to tetanus toxoid and greater proliferation of cord blood mononuclear cells in response to *Hp* than to tetanus (Malfitano et al., 2006).

To investigate the unexpected lower proliferative response of PBMCs from *Hp*+ compared to *Hp*- patients Jakob *et al.* postulated the *Hp* infection lead to "downregulated immunoreactivity". They stimulated PBMCs from *Hp*-infected patients with both their own *Hp* (autologous strain) and *Hp* isolated from other patients (heterologous strains). Stimulation with autologous *Hp* lead to lower proliferation but higher IL-10 production compared to stimulation with heterologous strains (Jakob et al., 2001). In support of this Kayhan *et al.* found higher IL-10 levels in the serum of *Hp*+ patients compared to uninfected healthy controls (Kayhan et al., 2008). This study also found evidence of an innate response to *Hp* in the peripheral blood with neutrophils and $\gamma\delta$ T cells increased in *Hp*+ patients compared to uninfected controls (Kayhan et al., 2008).

4.1.2 Peripheral Blood Treg Response to *Hp*

Work by others in the research group has shown increased levels of $CD4^+CD25^{hi}IL\text{-}10^+$ cells in peripheral blood of *Hp*+ compared to *Hp*- patients (Kenefeck et al., 2007), indicating that Tregs are an important source of the suppressive cytokine IL-10. Lundgren *et al.* found that depleting $CD25^{high}$ cells from $CD4^+$ memory cells of *Hp*+ donors increased their proliferative response to *Hp*-pulsed DCs but did not affect their response to tetanus toxoid-pulsed DCs. Co-culture experiments confirmed that $CD25^{high}$ cells reduced memory T cell proliferation and IFNγ secretion in response to *Hp*-pulsed DCs (Lundgren et al., 2003).

In addition to $CD4^+CD25^{high}IL\text{-}10^+$ cells others in the research group have shown increased proportions of CTLA-4^+ Tregs in the peripheral blood of *Hp*-infected patients compared to controls. Occurence of *Hp*-associated peptic

ulcer disease was associated with lower frequencies of CTLA-4$^+$ and IL-10$^+$ CD4$^+$CD25^{high} Tregs (Greenaway et al., 2011).

Satoh *et al.* also found increased CD4$^+$CD25^{high} Tregs, but no change in CD4$^+$CD25^{high}FOXP3$^+$ cell frequencies in the blood of *Hp*-infected patients (Satoh et al.).

There is interest in the hypothesis that systemic Treg responses to *Hp* could lead to protection against allergy and autoimmunity (see section 1.1.3.5).

4.1.3 Peripheral Blood Th1 and Th2 Responses to *Hp*

Ren *et al.* found an increased IFNγ response to *Hp* antigen upon stimulation of whole blood from *Hp*+ patients compared to uninfected controls (Ren et al., 2000). Quiding-Jarbrink *et al.* isolated peripheral blood T cells, stimulated with *Hp* antigen, and found increased IFNγ secretion and increased frequencies of IFNγ$^+$ T cells in *Hp*+ subjects compared to uninfected controls, though some uninfected controls did have a low level IFNγ response to *Hp* antigen stimulation. It was noted that there was wide individual variation in IFNγ response. Separation of CD4$^+$ T cells and CD8$^+$ T cells indicated that there were higher levels of IFNγ and increased frequencies of IFNγ$^+$ cells amongst the CD8$^+$ T cells compared to the CD4$^+$ T cells (Quiding-Jarbrink et al., 2001b). However, an earlier study found that HLA-DR antibody blocked the proliferative response to *Hp,* indicating the importance of CD4$^+$ cells here (Birkholz et al., 1993). CD8$^+$ T cells are reduced in the blood of *Hp*-infected patients, leading to an increased CD4:CD8 T cell ratio (Satoh et al.), as noted in the *Hp*-infected gastric mucosa (Figure 6.3A).

Ren *et al.* investigated the blood of *Hp*-infected patients following *Hp* eradication and found that the levels of IL-4 increased, though IFNγ levels were unchanged, suggesting a readjustment of the Th1/Th2 balance (Ren et al., 2000). Quiding-Jarbrink found that IL-4 and IL-5 could rarely be detected following 48 hours of *Hp* antigen stimulation (Quiding-Jarbrink et al., 2001b). In contrast Satoh *et al.* found no difference in CD4$^+$IFNγ$^+$ cells in the blood of their *Hp*-infected patients and uninfected control group with increased

$CD4^+IL\text{-}4^+$ cells in the *Hp*-infected group (Satoh et al.). Mitogen stimulation with PMA/ionomycin was used in this study whereas *Hp* antigen was used by Ren and Quiding-Jarbrink.

4.1.4 Peripheral Blood IL-17/Th17 Responses to *Hp*

Jafarzadeh *et al.* report increased serum IL-17 levels in *Hp* patients with duodenal ulcer compared to both healthy controls and asymptomatic *Hp* serology-positive participants. In this study those positive for anti-*cagA* antibodies in the *Hp*+ duodenal ulcer ulcer group had higher serum IL-17 levels than Cag A seronegative *Hp*+ duodenal ulcer patients (Jafarzadeh et al., 2009).

A recent study assessed Th17 cell frequencies in 3 groups of patients: those with current *Hp* infection, those with previous infection (no evidence of current infection but a history of *Hp* eradication or positive serology) and those that had never been infected with *Hp*. They found increased Th17 levels in the previous infection group compared to the *Hp* naive group, but the levels in the active *Hp* infection group were not significantly different to the other two groups (Serelli-Lee et al., 2012). HLA-DR blocking antibody substantially reduced IL-17 secretion by blood-derived $CD4^+$ T cells incubated with *Hp*-pulsed antigen presenting cells (Serelli-Lee et al., 2012).

I am not aware of any other studies of peripheral blood Th17 responses to *Hp* but there are reports of peripheral IL-17 responses to other infections, including viral infections such as chronic hepatitis B (Ge et al., 2009) where it positively correlated with viral load and histological activity index (Zhang et al.) and HIV where reduced Th17:Treg ratio was associated with disease progression (Chauhan et al., 2009) and an expanded Vδ1 IFNγ and IL-17 producing population was identified (Fenoglio et al., 2009). Circulating $CD4^+IL\text{-}17^+$ cells have also been identified in *C. albicans* infection (Zhou et al., 2008b) and patients suffering with the condition chronic mucocutaneous candidiasis have been found to have defects in Th17 immunity including STAT3

deficiency, IL-17 receptor A deficiency and IL-17F deficiency (Puel et al., 2011, Eyerich et al., 2008).

4.1.5 Modulation of Activation Markers on PBMCs by *Hp*

The response of a number of activation markers to *Hp* infection has been reported in the literature. Studies that have compared activation marker levels in the blood of *Hp*-infected and uninfected subjects have generally not found a significant difference: Kayhan *et al.* found no increase in HLA-DR on T cells in 32 *Hp*+ patients compared to 15 uninfected patients (Kayhan et al., 2008). Soares *et al.* agreed that there was no difference in T cell HLA-DR between infected and uninfected patients but found increased $CD4^+$ T cells in infected patients and an increased percentage of the costimulatory molecule CD28 on both $CD4^+$ and $CD8^+$ T cells (Soares et al., 2005). In contrast studies where PBMCs have been stimulated with *Hp* antigen *in vitro* have found that activation markers rise. Jakob *et al.* found increased CD25, CD71 and HLA-DR on both $CD4^+$ and $CD8^+$ T cells following 6 days of exposure to *Hp* antigen (Jakob et al., 2001). Similarly Kartunnen *et al.* reported increased CD25 and HLA-DR expression on $CD4^+$ and $CD8^+$ T cells after 7 days of stimulation with a 6 strain mix of *Hp* antigen (Karttunen, 1991).

4.1.6 Markers for Antigen-Specific Peripheral Blood $CD4^+$ T Cell Responses

As there are circulating lymphocytes with a wide range of specificities in the peripheral blood, activation markers are sometimes used to select cells specific for the stimulus used. Cells gated for the activation marker can then be used for subsequent analysis. My colleagues have used the early activation marker CD69 for this purpose (Robinson et al., 2002). CD154, also known as CD40 ligand, is another activation marker, specific for $CD4^+$ T cells. It interacts with CD40 on antigen presenting cells forming a costimulatory pair. It is thought to be more specific than other activation markers, such as CD25, CD69 and CD71 which can become activated in the absence of TCR engagement (Frentsch et al., 2005).

There is no single marker that definitively identifies Tregs in humans. Tregs are heterogeneous. Many Treg markers, including CD25, GITR and FOXP3 can also be transiently expressed on activated human T cells (Wang et al., 2007a). The transcription factor Helios was proposed as a marker to differentiate between thymus-derived "natural" Tregs and peripheral induced Tregs (Thornton et al., 2010) but more recently it has also been found on activated and proliferating $CD4^+$ and $CD8^+$ human T cells (Akimova et al., 2011). Surface staining for $CD4^+CD25^{high}CD127^{lo}$ has been widely used to identify human Tregs (Liu et al., 2006) and this profile is also able to distinguish Tregs from activated T cells (Seddiki et al., 2006). This combination of markers was used here along with FOXP3 staining.

Th1 and Th17 cells were identified as $CD4^+$ cells staining for IFNγ and IL-17 respectively. An alternative strategy would have been to stain for the transcription factors T-bet and RORC2, but non-specific staining was found to be problematic using this approach. $CD4^+$ T cells responding to *Hp* antigen stimulation with cytokine secretion would be expected to reflect the functional T helper response to the pathogen. As IFNγ and IL-17 were included in the same tubes it was also possible to identify $CD4^+$IL-17^+IFNγ^+ "double-positive" responders. This double-positive population is thought to be Th17-derived, pro-inflammatory and pathogenic and is discussed in section 1.3.3 and section 4.4.2 below (Peters et al., 2011, Hirota et al., Abromson-Leeman et al., 2009, Nistala et al., 2010).

4.2 AIMS

i. To compare serum IL-17 levels in *Hp* infected patients and uninfected controls.

ii. To optimize peripheral blood antigen-specific Th1 and Th17 staining and to measure and compare systemic Th1 and Th17 responses to *Hp.*

iii. To characterize the peripheral blood Treg response to *Hp* infection.

iv. To correlate peripheral blood Th1, Th17 and Treg responses with occurrence of *Hp*-associated disease.

v. To correlate the peripheral blood Th1 and Th17 responses with patient age and gender.

4.3 RESULTS

4.3.1 Serum IL-17 Measurement

Attempts were made to measure IL-17 levels in serum from 14 *Hp*+ patients and 13 uninfected controls. IL-17 levels in all the sera were below the limit of sensitivity of the ELISA (calculated as 7.0pg/ml and 8.1pg/ml for the 2 plates). The lowest standard was 6.25pg/ml IL-17. For the first assay samples were incubated on the plate for 2 hours at room temperature, as per standard ELISA protocol. For the second assay the samples were incubated overnight on the plate at 4°C as the ELISA kit instructions recommended that this would maximize sensitivity, however IL-17 levels remained below the limit of detection.

4.3.2 Optimization of Peripheral Blood Antigen-Specific T Helper Staining

As T cells in the blood have a wide range of specificities, staining for an activation marker such as CD69 or CD154 upon antigen stimulation may be used to select antigen specific cells, either alone or in combination with cytokine staining (Grabowska et al., 2001, Robinson et al., 2002, Frentsch et al., 2005, Morgan et al.). Tetramers staining is an alternative method for identifying antigen-specific cells (see discussion, section 4.4.1).CD69 and CD154 activation markers were first compared using PBMCs from a healthy donor with tetanus toxoid as an antigenic stimulus.

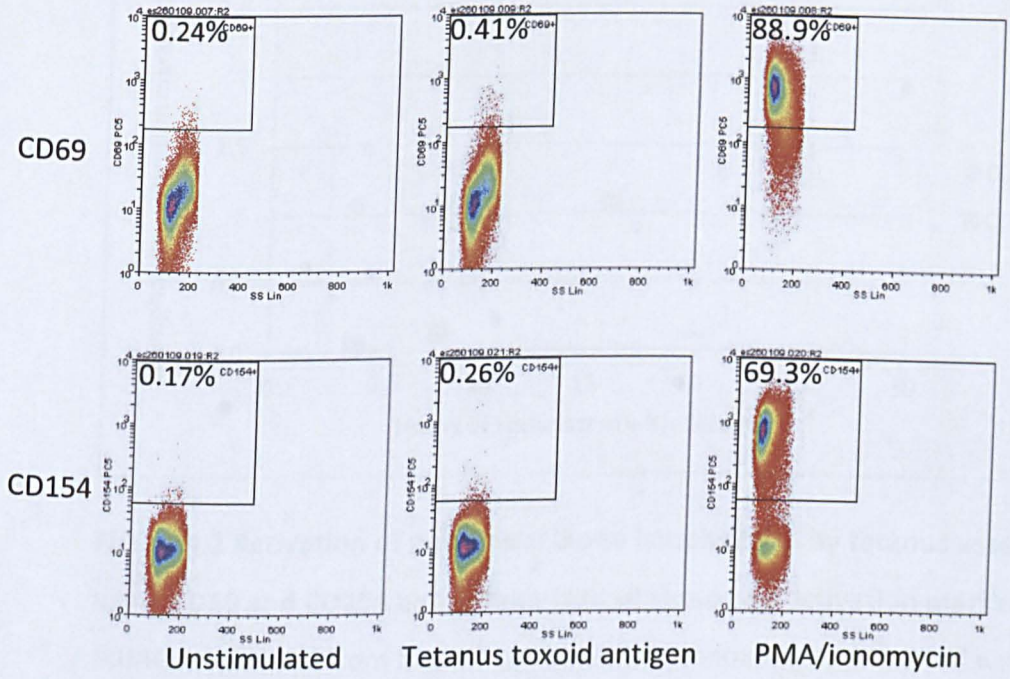


Figure 4.1 Example plots showing CD69 and CD154 staining with antigen and mitogen stimulation. PBMCs were stimulated with medium only (unstimulated control), tetanus toxoid antigen or PMA and ionomycin for 16 hours and labelled with the fluorescence-conjugated antibodies CD4-ECD and CD154-PC5 or CD69-PC5.

Antigen stimulation caused only a small amount of lymphocyte activation as measured by CD69 and CD154. The lymphocyte population divided into two subpopulations when stimulated with PMA/ionomycin and stained with CD154, as CD154 is only present on activated $CD4^+$ T cells. This facilitated identification and gating of the $CD154^+$ population. In contrast, the whole lymphocyte population increased its CD69 expression when stimulated with PMA/ionomycin (Figure 4.1).

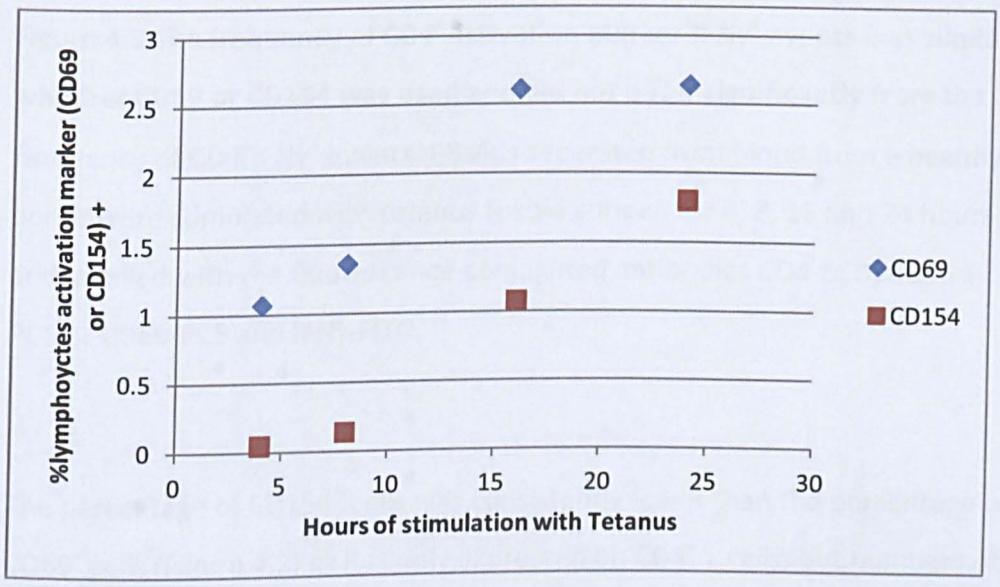


Figure 4.2 Activation of peripheral blood lymphocytes by tetanus assessed using CD69 and CD154 and comparison of these two activation markers. PBMCs separated from blood from a healthy donor were stimulated with tetanus toxoid antigen for 4, 8, 16 and 24 hours and labelled with the fluorescence-conjugated antibodies CD4-ECD and CD154-PC5 or CD69-PC5.

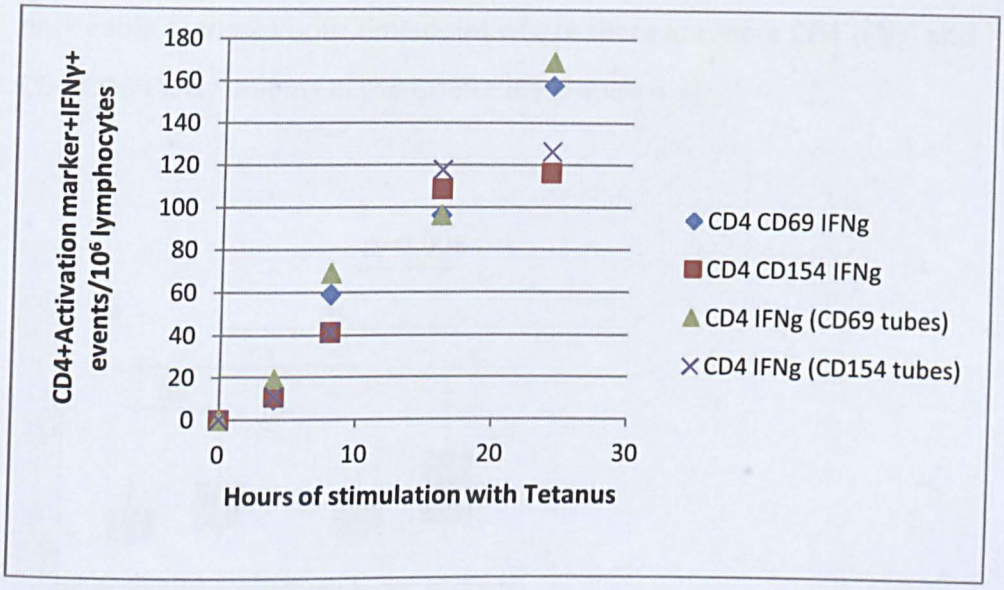


Figure 4.3 The frequency of $CD4^+$Activation $marker^+IFN\gamma^+$ events was similar whether CD69 or CD154 was used and did not differ significantly from the frequency of $CD4^+IFN\gamma^+$ events. PBMCs separated from blood from a healthy donor were stimulated with tetanus toxoid antigen for 4, 8, 16 and 24 hours and labelled with the fluorescence-conjugated antibodies CD4-ECD, CD154-PC5 or CD69-PC5 and IFNγ-FITC.

The percentage of $CD154^+$ cells was consistently lower than the percentage of $CD69^+$ cells (Figure 4.2) as it is only expressed on $CD4^+$ T cells, but numbers of $CD4^+$cytokine$^+$ events were very similar for the two activation markers. If no activation marker was used and $CD4^+IFN\gamma^+$ lymphocytes were analyzed the results also correlated well (Figure 4.3).

Ideally for this comparison CD69 and CD154 should have both been in the same tubes, but it was planned to use the same fluorochrome for whichever activation marker was decided upon so this was not feasible. $CD4^+IFN\gamma^+$ events were analyzed without activation marker gating in two tubes for each time point (the tube containing CD69 and the tube containing CD154). The tubes were prepared from the same donor at the same time so theoretically they should give the same results but there is a little variation, most

noticeable at the 24 hour time point where there are more $CD4^+IFN\gamma^+$ and $CD4^+CD69^+IFN\gamma^+$ events in the CD69 tube (Figure 4.3).

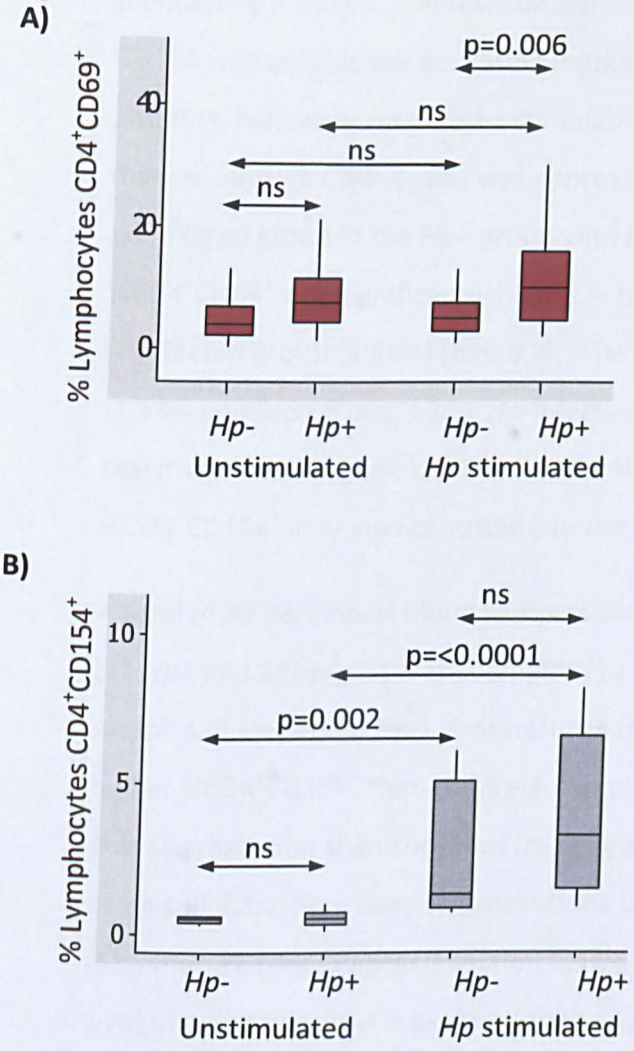


Figure 4.4 Activation of peripheral blood lymphocytes by *Hp* assessed using CD69 and CD154. PBMCs were incubated with medium only or *Hp* whole cell sonicate isolated from 6 clinical strains for 16 hours, then labelled with the fluorescence-conjugated antibodies CD4-ECD and CD69-PC5 or CD154-PC5. Frequency of $CD4^+$activation $marker^+$ events was then analyzed. A) CD69 activation marker. 20 *Hp* infected and 42 uninfected patients. B) CD154 activation marker. 13 *Hp* infected and 7 uninfected patients.

The baseline levels of CD154 in the unstimulated samples were lower than the CD69 levels (1.33% vs 4.88%) (Figure 4.4). As a result levels were significantly increased in cells stimulated with *Hp* whole cell sonicate (containing 6 clinical isolates) compared to unstimulated samples when CD154 was used as the activation marker for both the *Hp*+ and uninfected patients. Following *Hp* antigen stimulation the percentage of activation marker positive $CD4^+$ T cells was approximately double that found in the uninfected group in the *Hp*+ group with both activation markers. Median $\%CD4^+CD69^+$ was significantly higher in the *Hp*+ patients (10.14%) than in the uninfected group (4.88%) (p=0.006). The $\%CD4^+CD154^+$ events were lower (1.33% uninfected and 3.28% *Hp*-infected patients) and the p value did not reach significance (p=0.11). The smaller numbers of samples analyzed for $\%CD4^+CD154^+$ may also contribute to the higher p value.

A total of 34 peripheral blood samples were processed with CD154 staining (21 *Hp*+ and 13*Hp*-): 21 fresh samples (14 *Hp*+ and 7 *Hp*-) and 13 frozen samples (7 *Hp*+ and 6 *Hp*-). Amongst the *Hp*- patients the frozen samples had higher $\%CD4^+CD154^+$ than the fresh samples (median 6.92 vs 1.33, p=0.038). This suggests that the process of freezing and thawing the samples caused some cell activation, despite precautions taken to minimize rapid changes in temperature (see Chapter 2 section 2.5.2), thereby increasing the background level of activation. This is likely to mask any increase in activation with *Hp* stimulation. There was no significant difference between $\%CD4^+CD154^+$ levels for the *Hp*+ fresh and frozen samples. There was no significant difference in frequencies of $CD4^+IL\text{-}17^+$ or $CD4^+IFN\gamma^+$ events between the fresh and frozen samples in either the *Hp*+ or *Hp*- groups suggesting that CD154 is more susceptible to increase on freeze-thawing than the cytokines.

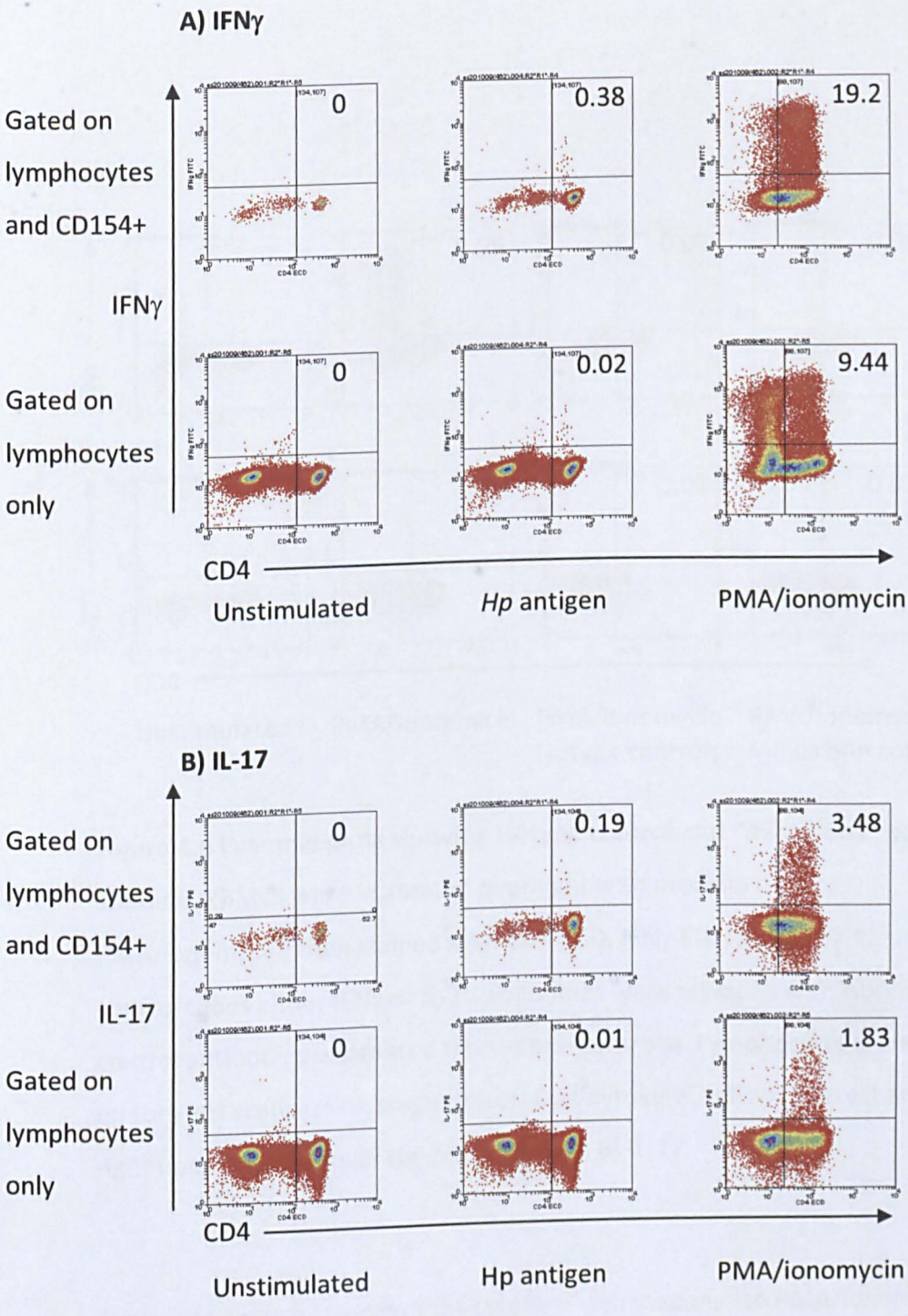


Figure 4.5 Example plots comparing analysis of $CD4^+$cytokine$^+$ events with lymphocyte and CD154 gating or lymphocyte gating only. PBMCs were incubated overnight with medium only, *Hp* antigen or PMA/ionomycin, then stained with CD4-ECD, CD154-PC5, IFNγ-FITC and IL-17-PE. Data was analyzed using both gating strategies for A) $CD4^+IFN\gamma^+$ and B) $CD4^+IL\text{-}17^+$ events.

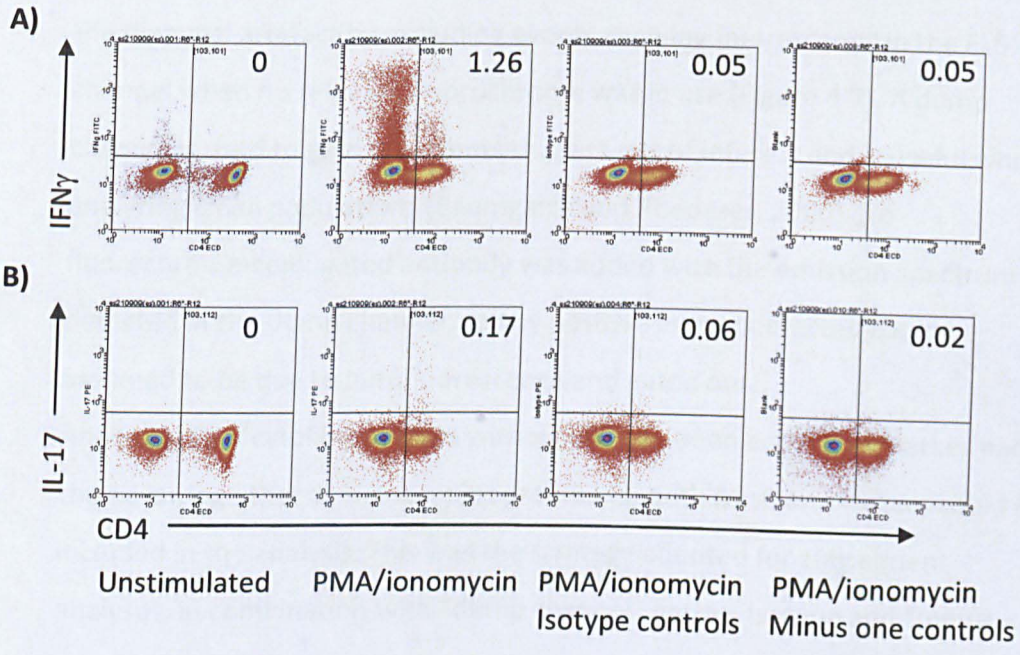


Figure 4.6 Example plots showing isotype control and "minus one" control staining. PBMCs were incubated overnight with medium only or PMA/ionomycin, then stained with CD4-ECD, IFNγ-FITC and IL-17-PE. In control tubes either IFNγ or IL-17 antibodies were replaced with isotype control antibodies or omitted (minus one controls). Lymphocytes were gated on forward scatter/side scatter plots. $CD4^+$cytokine$^+$ cells of interest are in the right upper quadrants of the plots. A) IFNγ, B) IL-17.

There was a much stronger $CD4^+$cytokine$^+$ cell response to PMA/ionomycin than to *Hp* antigen, as expected. The $CD4^+IFN\gamma^+$ response to PMA/ionomycin was stronger than the $CD4^+IL\text{-}17^+$ response. Use of an activation marker had little effect on the numbers of $CD4^+$cytokine$^+$ events detected, though it did reduce the diagonal artefact sometime found when cells were gated on lymphocytes only (Figure 4.5). Despite the relatively long (16 hour) stimulation with PMA/ionomycin cell death, as assessed on forward

scatter/side scatter plots, did not appear to be a significant issue. A specific stain to assess viability was not used in this study. Most of the diagonal artefact was in the CD4- negative part of the plot and therefore excluded from the analysis. A "dump channel" strategy was used also used to reduce the diagonal artefact by excluding events showing fluorescence in the FL5 channel when no relevant fluorochrome was in use (Figure 4.7). A dump channel is used to gate out events that are not of interest and is useful when analyzing small populations (Baumgarth and Roederer, 2000). No fluorochrome-conjugated antibody was added with the emission spectrum detected in the dump channel, so any positive events detected could be assumed to be due to autofluorescence and gated out.

Analyzing $CD4^+$cytokine$^+$ events without the use of an activation marker had the advantage that all the samples processed, both fresh and frozen, could be included in the analysis. This was the strategy adopted for subsequent analyses, in combination with "dump channel" gating. Isotype and "minus one" controls for IFNγ and IL-17 staining are shown in Figure 4.6.

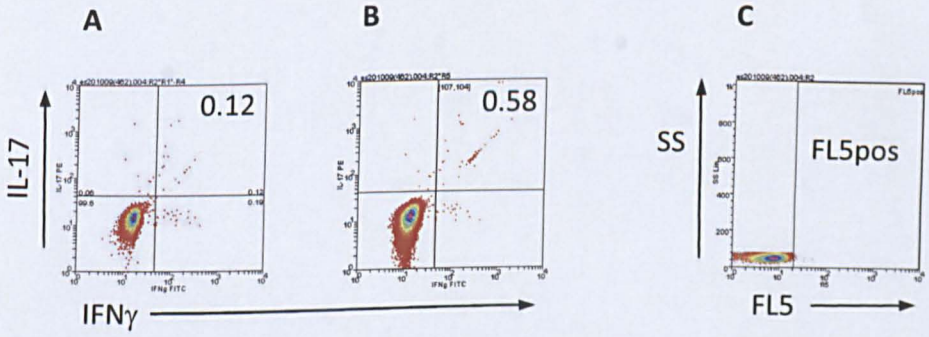


Figure 4.7 Dump channel used to gate out autofluorescence to minimise artefactual double positive staining. PBMCs were incubated for 16 hours with *Hp* antigen, labelled with the fluorochrome-conjugated antibodies CD4-ECD, IFNγ-FITC and IL-17-PE and analyzed by flow cytometry.

A) Cells gated on lymphocytes, $CD4^+$ and dump channel ("NOT FL5pos").

B) Cells gated on lymphocytes and $CD4^+$ only.

C) FL5pos region determined on FL5 vs side scatter plot.

Representative example from an *Hp+* donor.

4.3.3 The Systemic Th1 and Th17 Response to *Hp*

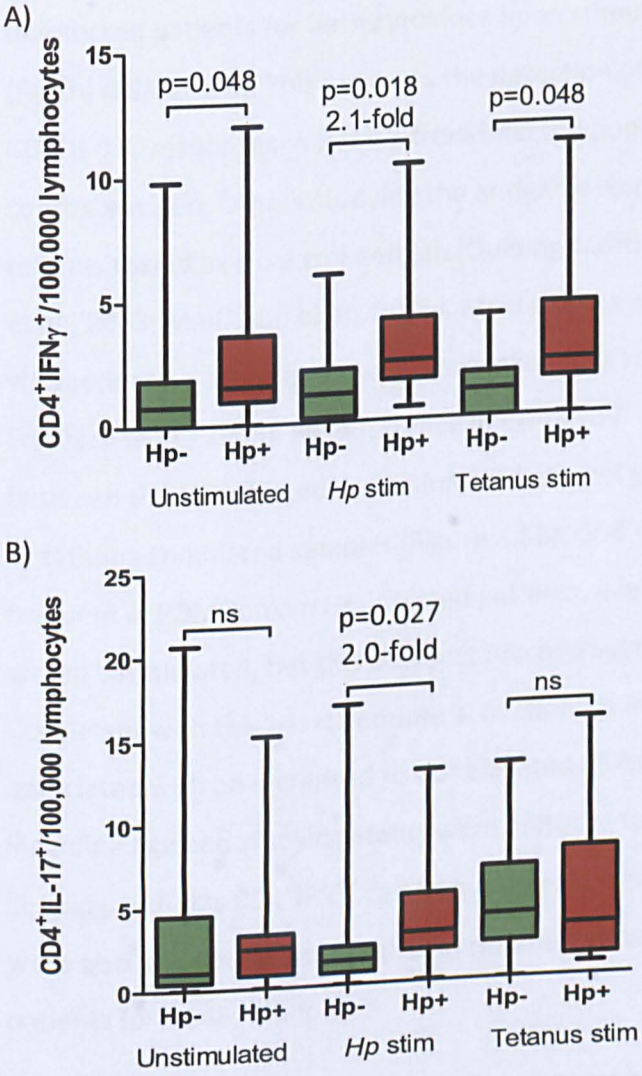


Figure 4.8 The systemic Th1 and Th17 response to *Hp* and tetanus. PBMCs were incubated overnight with medium only, *Hp* antigen (21 *Hp*+ and 13*Hp*-) tetanus toxoid (16*Hp*+ and 12*Hp*-), then labelled with the fluorochrome-conjugated antibodies CD4-ECD, IFNγ-FITC and IL-17-PE. Cells were gated on lymphocytes and autofluorescent cells gated out using a dump channel. $CD4^+$cytokine$^+$events per 100,000 lymphocytes were analyzed. A) $CD4^+IFN\gamma^+$/100,000 events, B) $CD4^+IL\text{-}17^+$/100,000 events. Boxes represent the 25th to 75th centiles, horizontal lines within the boxes represent the median and the whiskers depict the 5th and 95th centiles.

Although the absolute numbers of events were not very high there were significant differences in the $CD4^+$cytokine$^+$ events between *Hp*-infected and uninfected patients for both cytokines upon stimulation with *Hp* antigen (Figure 4.8A and B). This indicates the detection of *Hp*-specific $CD4^+IFN\gamma^+$ and $CD4^+IL\text{-}17^+$ responses in PBMCs from infected donors. Tetanus was used as a control antigen. Others studying the antigenic response to *Hp* have used tetanus toxoid as a control antigen (Quiding-Jarbrink et al., 2001a, Lundgren et al., 2003, Malfitano et al., 2006). Most donors will have been exposed to it via vaccination and it is known to provoke a Th1 response (Viana et al., Frentsch et al., 2005). No difference in $CD4^+IL\text{-}17^+$ frequencies was seen between the *Hp*-infected and uninfected control groups for the unstimulated or tetanus stimulated samples (Figure 4.8B). $CD4^+IFN\gamma^+$ events were more frequent in PBMCs from *Hp*-infected patients, even when the samples weren't stimulated, but this only just reached significance (Figure 4.8A). Consistent with this basal immune activation *Hp* infection was found to be associated with an increased risk of elevated CRP (>3 mg/L) when factors including age and smoking status were adjusted for (Jackson et al., 2009). In keeping with this $CD4^+IFN\gamma^+$ cell frequencies in tetanus stimulated samples were also just significantly higher in *Hp*-infected compared to uninfected patients (p=0.048, 1.6-fold).

For 2 patients (1 *Hp*+ and 1 with positive *Hp* serology but negative rapid, urease test, histology and culture) response to stimulation with *C. albicans* was also assessed by flow cytometry, as most donors are likely to have been exposed to *C. albicans* and it is known to provoke a Th17 response (Zhou et al., 2008b, Zielinski et al., 2012). The *Hp*+ patient had 4.74 and 1.09 $CD4^+IL\text{-}17^+$ cells/100,000 lymphocytes following PBMC stimulation with *Hp* and *Candida* respectively. The other patient, with probable previous *Hp* infection, had 1.60 and 1.81 $CD4^+IL\text{-}17^+$ cells/100,000 lymphocytes following PBMC stimulation with *Hp* and candida respectively. Both absolute numbers of events of interest and number of patients studied here was low making it unjustified to draw any conclusions about the exact numbers found, but these

findings suggest that the IL-17 response of PBMCs to *Hp* is of a similar magnitude to their response to *C. albicans.*

Mitogen stimulation with PMA and ionomycin resulted in much higher frequencies of $CD4^+$cytokine$^+$ events. $CD4^+IFN\gamma^+$ cells were increased in *Hp*-infected compared to uninfected patients upon PMA/ionomycin stimulation (p=0.0009, median $CD4^+IFN\gamma^+$/100,000 lymphocytes 8176 in *Hp*+ vs 3096 in *Hp*-). There was a trend for increased $CD4^+$IL-17^+ events in the *Hp*-infected group but this did not reach significance (p=0.15, median $CD4^+$IL-17^+/100,000 lymphocytes 528 in *Hp*+ vs 266 in *Hp*-).

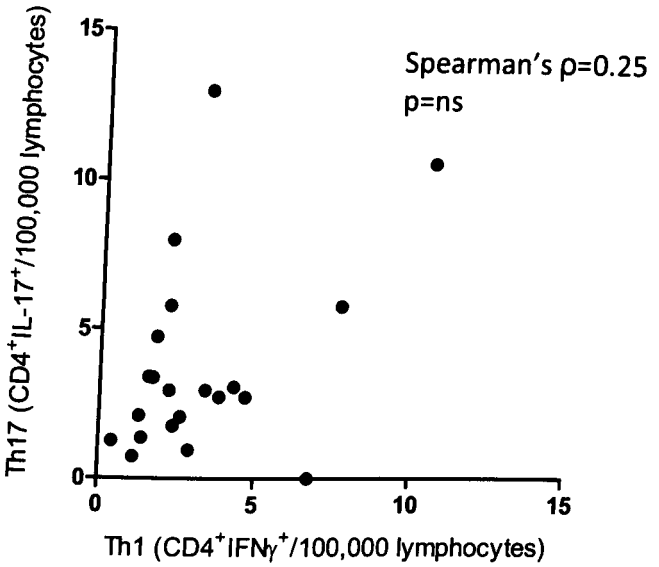


Figure 4.9 Correlation between $CD4^+IFN\gamma^+$ **and** $CD4^+IL\text{-}17^+$ **events.** PBMCs from 21 *Hp*+ donors were incubated with *Hp* antigen for 16 hours, then stained with fluorochrome conjugated antibodies CD4-ECD, IFNγ-FITC and IL-17-PE. Cells were gated on lymphocytes and autofluorescent cells gated out using a dump channel. $CD4^+IFN\gamma^+$events and $CD4^+IL\text{-}17^+$ events per 100,000 lymphocytes were analyzed and correlated.

There was no significant correlation between the numbers of $CD4^+IL\text{-}17^+$ and numbers of $CD4^+IFN\gamma^+$ cells found in the peripheral blood (Figure 4.9).

A proportion of $CD4^+IL\text{-}17^+$ cells also produced $IFN\gamma^+$. This ranged between 0 and 100%, with a median of 33.3% for the 21 *Hp*+ samples.

4.3.4 The Peripheral Blood Treg Response to *Hp*

Numerous markers have been used to identify Tregs. $CD4^{+}CD25^{high}$ and the transcription factor FOXP3 are probably the most widely used and accepted but often further markers are used in combination with these. I elected to use $CD127^{lo}$ in addition to $CD4CD25^{high}$ and FOXP3, as described by Liu *et al.* (Liu et al., 2006). $CD127^{lo}$ inversely correlates with FOXP3 but has the advantage that it is a surface marker meaning that it is possible to do further functional experiments with Tregs isolated on the basis of $CD4^{+}CD25^{high}CD127^{lo}$. CD127 is also a convenient marker to analyze as the cells generally separate into $CD127^{+}$ and $CD127^{lo}$ populations in contrast with some other markers such as CD25 which appears as a continuum in which a cut-off point must be established. A system of classifying human $FOXP3^{+}$ Tregs based on CD45RA and CD25 expression, including $CD25^{++}$ and $CD25^{+++}$ populations has also been described (discussed further in section 4.4.3). An example of $CD4^{+}CD25^{high}$ gating demonstrating the CD25 continuum is shown in Figure 4.10A. Figure 6.10B (already $CD4^{+}CD25^{high}$ gated) shows an example of gating of the $CD127^{lo}FOXP3^{+}$ population, which is easier to distinguish.

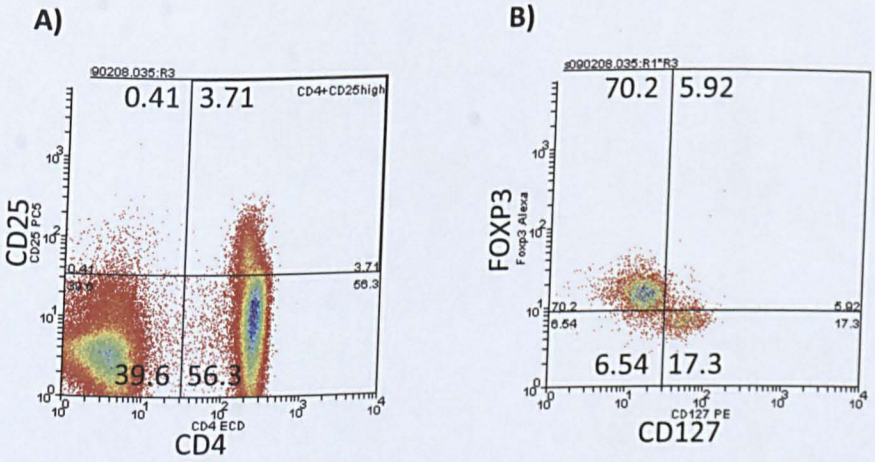


Figure 4.10 Example of Treg analysis gating strategy. PBMCs were cultured with *Hp* antigen for 16 hours and labelled with the fluorochrome-labelled antibodies CD4-ECD, CD25-PC5, CD127-PE and FOXP3-Alexa 488.

A) $CD4^{+}CD25^{high}$ gating,

B) $CD4^{+}CD25^{high}$ gated cells assessed for $CD127^{low}$ and $CD127^{low}FOXP3^{+}$.

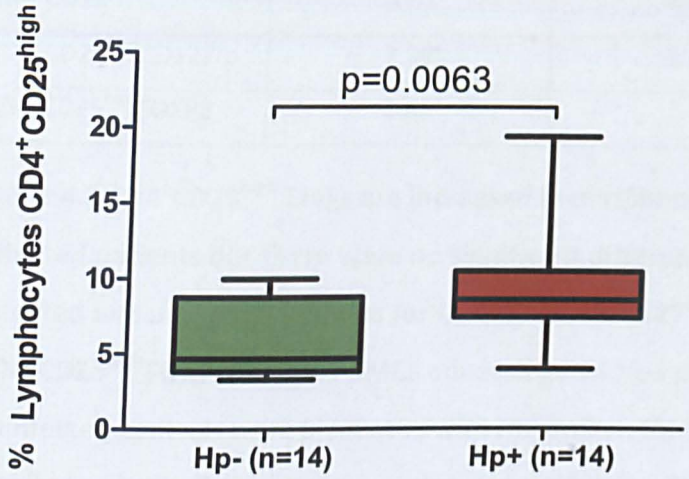


Figure 4.11 $CD4^+CD25^{high}$ Tregs are increased in peripheral blood of *Hp*-infected patients. PBMCs donated by 14 *Hp*+ patients and 14 uninfected controls were incubated with *Hp* antigen for 16 hours, then labelled with the fluorochrome-conjugated antibodies CD4-ECD and CD25-PC5. The %lymphocytes $CD4^+CD25^{high}$ in the infected and uninfected groups were compared.

	Hp-infected median	Uninfected median	P
$CD4^+CD25^{high}$	8.62	4.70	0.0063
$CD4^+CD25^{high}$CD127lo	2.89	2.41	0.22
$CD4^+CD25^{high}$FOXP3	2.85	2.15	0.26

Table 4.1 $CD4^+CD25^{high}$ Tregs are increased in peripheral blood of *Hp*-infected patients but there were no significant differences between the infected and uninfected groups for $CD4^+CD25^{high}CD127^{lo}$ or $CD4^+CD25^{high}FOXP3^+$ Tregs. PBMCs donated by 14 *Hp*+ patients and 14 uninfected controls were incubated with *Hp* antigen for 16 hours, then labelled with the fluorochrome-conjugated antibodies CD4-ECD, CD25-PC5, CD127-PE and FOXP3-Alexa488 and analyzed by flow cytometry. Median %lymphocytes positive for Treg markers for the *Hp*-infected and uninfected groups were calculated and compared.

$CD4^+CD25^{high}$ Tregs were significantly increased in the blood of *Hp*+ compared to uninfected patients (Figure 4.11 and Table 4.1) but there was no significant difference in the levels of $CD4^+CD25^{high}CD127^{lo}$ or $CD4^+CD25^{high}FOXP3^+$ Tregs (Table 4.1).

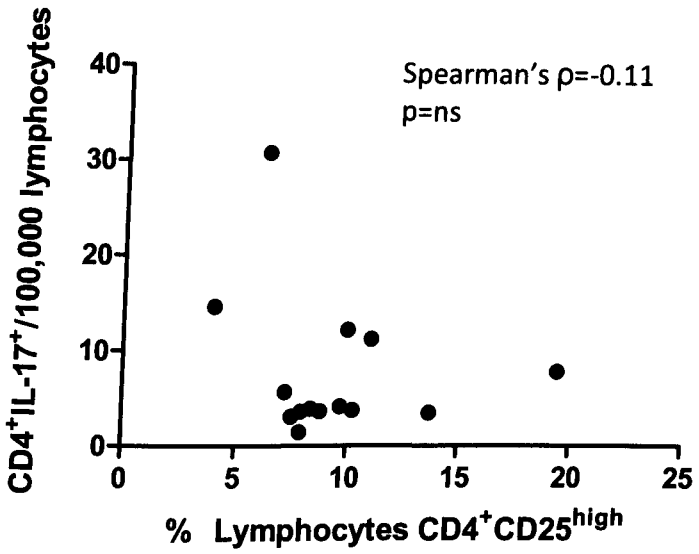


Figure 4.12 No correlation found between Treg and Th17 levels. PBMCs donated by 14 *Hp*+ patients were incubated with *Hp* antigen for 16 hours, then labelled with fluorochrome-conjugated antibodies to stain for Treg and Th17 markers, as above. Treg (% lymphocytes $CD4^+CD25^{high}$) and Th17 ($CD4^+IL\text{-}17^+$) frequencies were compared.

No significant correlation was found between levels of blood $CD4^+CD25^{high}$ Tregs and Th17 levels in *Hp*-infected patients (Figure 4.12).

4.3.5 Is There a Correlation Between the Peripheral Blood Th1, Th17 or Treg Response and Presence of Peptic Ulcer Disease?

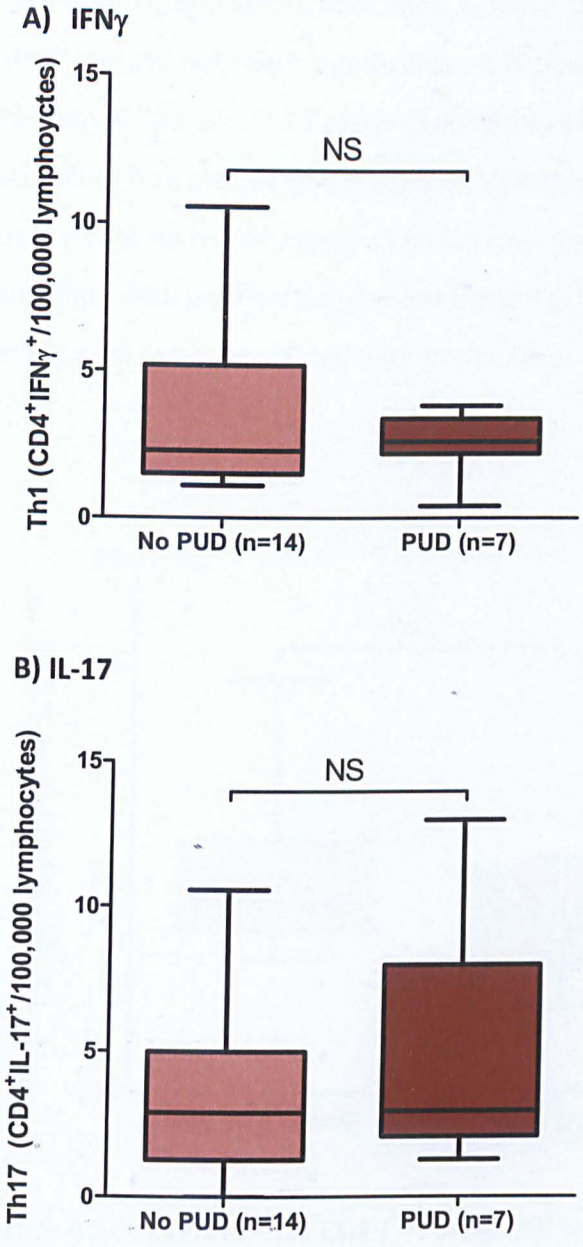


Figure 4.13 Correlation of Th1 and Th17 responses with occurrence of peptic ulcer disease. PBMCs from 21 *Hp*+ patients, 7 with peptic ulcer disease and 14 without, were incubated with *Hp* antigen for 16 hours, then stained with fluorochrome-conjugated antibodies CD4-ECD, IFNγ-FITC and IL-17-PE and analyzed by flow cytometry. Frequencies of A) $CD4^+IFN\gamma^+$ and B) $CD4^+IL\text{-}17^+$ events were compared in the peptic ulcer disease and ulcer-free groups.

The median peripheral blood Th1 ($CD4^+IFN\gamma^+$/100,000 lymphocytes) and Th17 ($CD4^+IL\text{-}17^+$/100,000 lymphocytes) responses both showed a trend of being higher in patients with peptic ulcer disease, but the differences were very small and did not reach significance. It is clear that there is a significant overlap of Th1 and Th17 systemic responses between the peptic ulcer disease and ulcer-free groups so it is unlikely that this type of data could give clinically useful data on risk of peptic ulcer disease. Neither did blood Treg levels correlate with peptic ulcer disease (Figure 4.14) though low gastric Treg levels are known to be associated with peptic ulcer disease (Robinson et al., 2008).

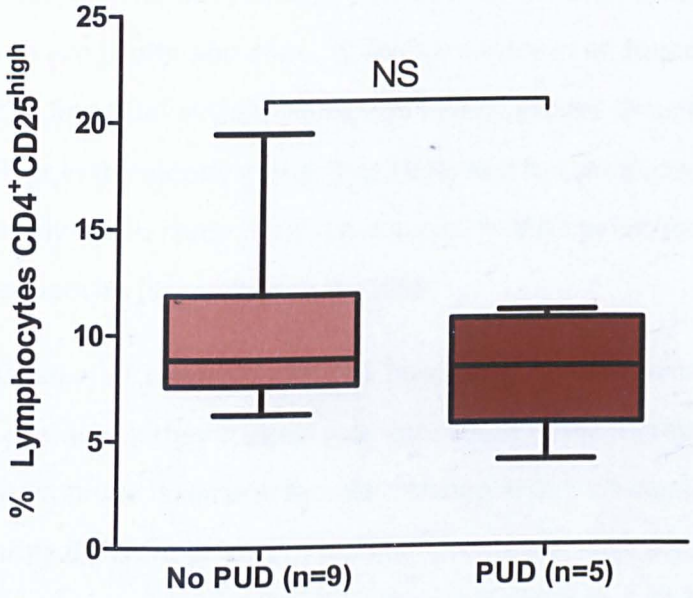


Figure 4.14 Levels of $CD4^+CD25^{high}$ Tregs did not correlate with peptic ulcer disease. PBMCs from 14 *Hp*+ donors, 5 with peptic ulcer disease and 9 without were incubated with *Hp* antigen for 16 hours, then stained with the fluorochrome-conjugated antibodies CD4-ECD and CD25-PC5 and analyzed by flow cytometry. % lymphocytes $CD4^+CD25^{high}$ in the peptic ulcer disease and ulcer-free groups were compared.

4.3.6 Correlation of Peripheral Blood Th1, Th17 and Treg Responses with Patient Age and Gender

Patients for this study were not selected at random from the general population but from those attending hospital for upper GI endoscopy. It was not possible to age- and sex-match the participants. In older individuals there is involution of the thymus and narrowing of the T cell repertoire with a higher proportion of antigen-experienced T cells and less naive T cells (Naylor et al., 2005, Arnold et al., Karanfilov et al., 1999). Ageing also has effects on T cell signalling, DNA repair and antioxidant mechanisms and leads to shortened telomeres (Arnold et al.). Hoffman *et al.* reported a positive correlation between age and intracellular IFNγ levels in $CD4^+$ T cells in children. In adults $CD4^+IL10^+$ and $CD8^+IFN\gamma^+$ but not $CD4^+IFN\gamma^+$ cells correlated with age (Hoffmann et al., 2005). Karanfilov *et al.* found similar frequencies of $CD4^+$ and $CD8^+$ in their young and elderly groups though the variation was wider in the elderly group. T cell IFNγ and IL-4 production was reduced in the elderly in this study but did not correlate with naive/memory T cell frequencies (Karanfilov et al., 1999).

Afshan *et al.* reported reduced Treg frequencies in females compared to males, which they suggest may contribute to the increased incidence of autoimmune disease in females (Afshan et al.). Changes in Treg numbers during different phases of the menstrual cycle have also been reported (Arruvito et al., 2007). In view of these findings Th1, Th17 and Treg levels were plotted against age and gender to ensure that these were not confounding factors.

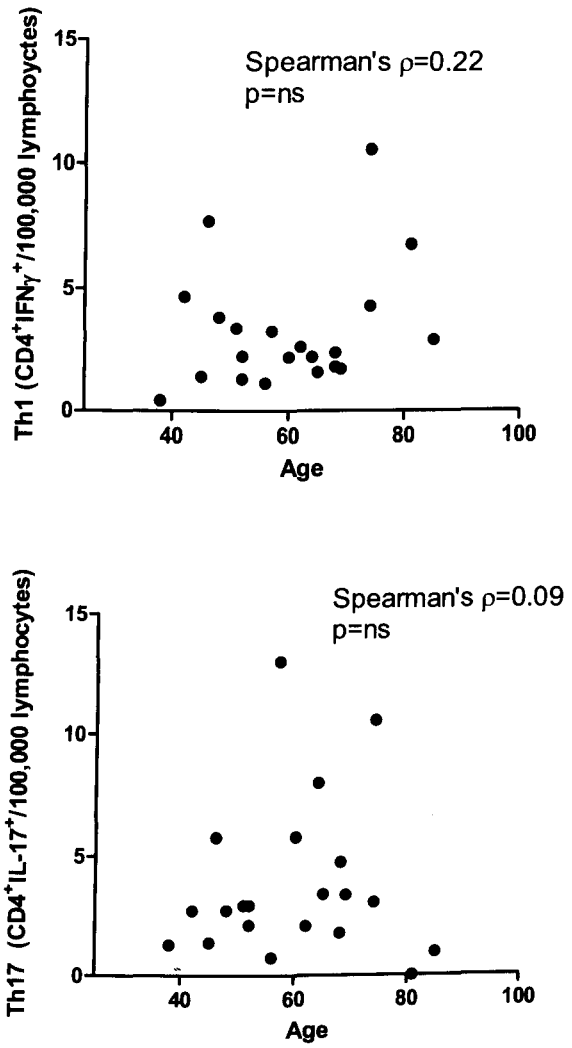


Figure 4.15 Th1 and Th17 cell frequencies did not correlate with age. PBMCs from 21 *Hp*+ donors were incubated with *Hp* antigen for 16 hours, then stained with fluorochrome-conjugated antibodies CD4-ECD, IFNγ-FITC and IL-17-PE. Cells were gated on lymphocytes and autofluorescent cells gated out using a dump channel. A) $CD4^+IFN\gamma^+$events and B) $CD4^+IL\text{-}17^+$ events per 100,000 lymphocytes were analyzed and correlated with patient age.

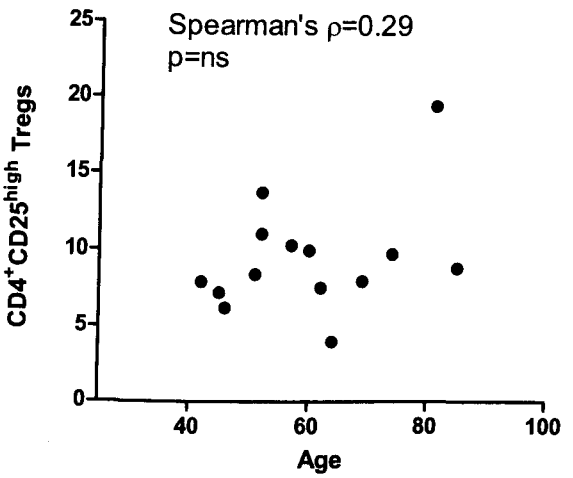


Figure 4.16 CD4$^+$CD25high Tregs levels did not correlate with age. PBMCs from 14 *Hp*+ donors were incubated with *Hp* antigen for 16 hours, then stained with the fluorochrome-conjugated antibodies CD4-ECD and CD25-PC5 and analyzed by flow cytometry. Results were correlated with the ages of the donors.

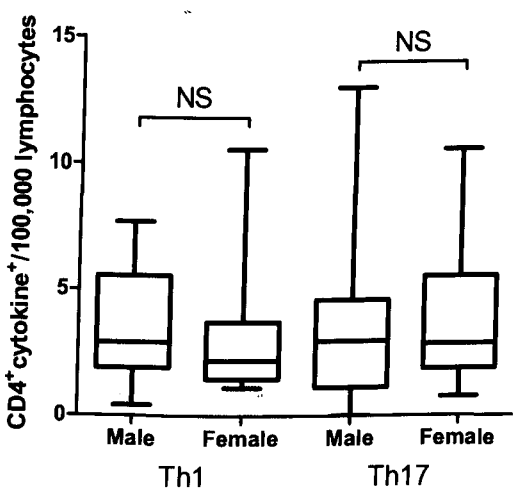


Figure 4.17 Th1 and Th17 levels in the peripheral blood were not associated with gender. PBMCs from 21 *Hp*+ donors (9 male and 12 female) were incubated with *Hp* antigen for 16 hours, then stained with the fluorochrome-conjugated antibodies CD4-ECD, IFNγ-FITC and IL-17-PE and analyzed by flow cytometry. Results for male and female donors were compared.

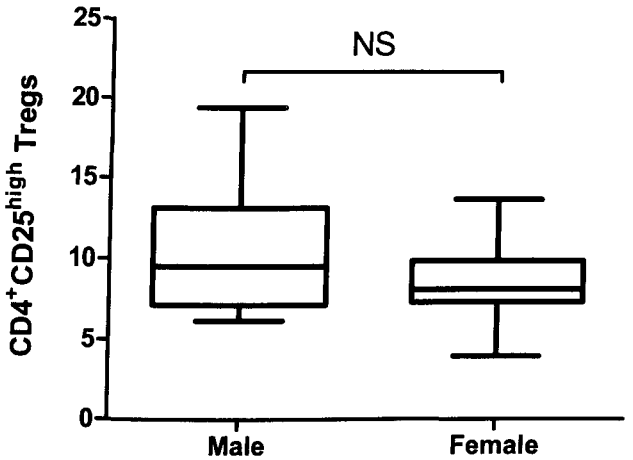


Figure 4.18 Treg levels in the peripheral blood were not associated with gender. PBMCs from 14 *Hp*+ donors (6 male and 8 female) were incubated with *Hp* antigen for 16 hours, then stained with the fluorochrome-conjugated antibodies CD4-ECD and CD25-PC5 and analyzed by flow cytometry. Results for male and female donors were compared.

There was no significant difference between the ages of the *Hp*+ and uninfected groups (median age of *Hp*+ group 60 years of age, median age of uninfected control group 59 years of age). Male:female ratios were similar with slightly more females in both groups (*Hp*+ group 9:12; *Hp*- group 5:8)

No correlation with age (Figure 4.15 and Figure 4.16) or gender (Figure 4.17 and Figure 4.18) was found for peripheral blood Th1, Th17 or Treg responses.

Thank you to Dr Rupert Kenefeck for additional $CD4^+CD69^+$ cell data.

4.4 DISCUSSION

4.4.1 Optimization of Peripheral Blood Antigen-Specific T Helper Staining

Initially CD69 and CD154 activation markers were compared, as they have been used to help identify antigen-specific cells in previous studies on peripheral blood (Robinson et al., 2002, Frentsch et al., 2005, Morgan et al., Grabowska et al., 2001). CD69 has been widely used as an early activation marker in the study of antigen-specific PBMC responses, including in a number of local studies (Robinson et al., 2002, Morgan et al., Grabowska et al., 2001). CD154 is also an early activation marker, reported to plateau 4-6 hours after stimulation *in vitro* (Frentsch et al., 2005). Unlike CD69 its expression is restricted to $CD4^+$ T cells. It is transiently expressed at the cell surface, then rapidly internalized, so intracellular staining is the method of choice for detection of low frequency antigen-specific $CD4^+$ T cells (Meier et al., 2008). Extracellular CD154 staining or inclusion of anti-CD154 antibody in the culture medium during stimulation can be used to identify viable antigen-specific $CD4^+$ T cells (Meier et al., 2008, Cohen et al., 2005, Chattopadhyay et al., 2005). CD154 is more specific for T cell receptor engagement that CD69 and is reported to be a robust marker for activation of T cells stimulated with peptides or protein (Frentsch et al., 2005, Meier et al., 2008). In keeping with the literature no difference was found in these activation markers on unstimulated PBMCs from *Hp*-infected and uninfected donors, but when the PBMCs were stimulated with *Hp in vitro* $CD4^+CD69^+$ levels were higher in the *Hp*-infected group, with a similar but not statistically significant trend for $CD4^+CD154^+$ lymphocytes (Figure 4.4). This indicates a *Hp*-specific peripheral blood $CD4^+$ T cell response in infected patients.

Levels of $CD4^+CD69^+IFN\gamma^+$, $CD4^+CD154^+IFN\gamma^+$ and $CD4^+IFN\gamma^+$ without the use of an activation marker were very similar. The vast majority of $IFN\gamma^+$ cells also expressed the activation markers (Figure 4.3). Cytokines may be considered a form of activation marker themselves, as they are usually only secreted when cells are stimulated. Others have studied the proportion of $CD154^+$ cells

secreting particular cytokines (Frentsch et al., 2005, Chattopadhyay et al., 2005). Not all $CD154^+$ cell secreted any of the cytokines measured, suggesting that these activated cells are working via other cytokines, mechanisms not involving soluble mediators, or reflect staining of non-activated cells. CD154 may be a good marker for a broad range of activated T cell functions but in this study IFNγ-secreting and IL-17-secreting cells were the particular focus.

MHC class II tetramers are an alternative technology that can be used to investigate antigen-specific $CD4^+$ T cell responses. This requires knowledge of the HLA-DR type of the donor and immunodominant peptide epitope(s) of the antigen of interest. Due to the low frequency of antigen-specific $CD4^+$ T cells in the peripheral blood it is normally necessary to either enrich or expand these cells *in vitro* (Cecconi et al., 2008). Bonvalet *et al.* expanded allergen-specific PBMC-derived $CD4^+$ T cells *in vitro*, then stained with tetramers and a panel of activation marker antibodies, including antibodies to CD69 and CD154. They found little correlation between tetramer and activation marker staining, with both $tetramer^-/marker^+$ and $marker^-/tetramer^+$ populations identified (Bonvalet et al.). The $tetramer^-/marker^+$ population secreted IL-5 and IL-10, but not as much as the $tetramer^+/marker^+$ cells. These cells may have become activated by cytokines secreted by allergen-specific $CD4^+$ T cells in a "bystander effect". This phenomenon has been described following tetanus toxoid booster vaccination (Di Genova et al.). The $marker^-/tetramer^+$ population is thought to represent an anergic or exhausted allergen-specific $CD4^+$ T cell subset (Bonvalet et al.).

As *Hp* antigen stimulation was used, it might be expected that only cells specific for *Hp* should be activated, but it is possible that epitopes in *Hp* antigen could activate cross-reactive T cell receptors or induce innate immunity via stimulation of PAMPs, leading to cytokine secretion and possible bystander activation of T cells with other specificities, as discussed above. *Hp*-specific memory T cells may be expected to account for a high proportion of the T cell recall response to *Hp* in *Hp*+ donors but evidence indicating that *Hp* can also activate naive T cells casts some doubt over this assumption

(Quiding-Jarbrink et al., 2001b, Malfitano et al., 2006). The expression of activation markers, but not cytokines was increased in frozen compared to fresh samples. Use of an activation marker was not felt to add anything and allowed combined analysis of fresh and frozen samples. This also allowed the possibility of analyzing all the samples stained with CD4 and IFNγ or IL-17 together, as the activation markers could be disregarded.

4.4.2 The Systemic Th1 and Th17 Responses to *Hp* Infection

Satoh *et al.* and Serelli-Lee *et al.* previously reported that there was no difference in the percentage of Th1 cells in the blood of *Hp*-infected patients compared to controls. Short (4-5 hour) stimulations with PMA/ionomycin were used in these studies. There were 45 *Hp*+ patients and 21 uninfected controls in Satoh's study and 37 *Hp*+ and 40 uninfected in Serelli-Lee's study, so it is surprising in view of the results presented here that no difference was found. The mitogen may have acted as such as strong stimulus on the blood of both groups that any differences were obscured. Studies by my colleagues using *Hp* antigen stimulation have previously shown increased blood $CD4^+CD69^+IFN\gamma^+$ cells and increased IFNγ secretion by PBMCs from *Hp*+ donors compared to PBMCs from uninfected controls (Kenefeck, 2008). Ren *et al.* and Quiding-Jarbrink *et al.* also found increased IFNγ secretion from whole blood and peripheral blood T cells of *Hp*+ donors upon *Hp* antigen stimulation, in keeping with the findings presented here (Ren et al., 2000, Quiding-Jarbrink et al., 2001b).

This is the first study to demonstrate a systemic Th17 response to *Hp* in those with current infection. Serelli-Lee *et al.* measured the percentage of $CD3^+CD8^-CCR6^+IL\text{-}17^+$ cells as a function of $CD3^+CD8^-$ PBMCs following 5 hours PMA/ionomycin stimulation to quantify Th17 cells in the blood. PMA causes downregulation of CD4 expression (Pelchen-Matthews et al., 1993) so it is easier to gate $CD3^+CD8^-$ PBMCs than $CD4^+$ T cells directly following PMA stimulation. $CD3^+CD8^-$ cells were assumed to be $CD4^+$ T cells. In this study median Th17 levels were significantly higher in the previously *Hp*-infected group (1.4%) compared to the *Hp* naive group (0.8%), suggesting that the

Th17 response to *Hp* persists after the infection is cleared. The median of the group with current *Hp* infection (1.1%) was not significantly different to either of the other two groups. As PMA/ionomycin is a non-specific stimulus it is possible that Th17 cells with other specificities were reactivated in this study. The level of Th17 response found here correlates with the ages of the patients in the different groups with mean patient age lowest in the *Hp* naive group (51), higher in the current *Hp* infection group (56) and highest in the previous infection group (59) (Serelli-Lee et al., 2012). Older patients may have had more pathogen exposure in general and hence have more Th17 cells that can reactivate in response to PMA/ionomycin stimulation. However in the same study levels of IL-17 secreted by *Hp*-stimulated PBMCs from the three groups and levels of $CD4^+IL\text{-}17^+$ cells in snap frozen gastric biopsies by immunofluorescence microscopy showed similar patterns (Serelli-Lee et al., 2012).

The Th17 response to *Hp* detected here was of similar magnitude to the Th1 response. The absolute numbers of events was low but consistent with the low frequency of antigen-specific $CD4^+$ T cells found in peripheral blood in other studies (Frentsch et al., 2005, Zhou et al., 2008b). To try to maximize the number of events of interest 400,000 events were typically acquired. Other ways to increase the number of $CD4^+$ T cells staining for cytokine would have been to expand the PBMCs with longer-term antigen stimulation *in vitro* or to use mitogen stimulation. This type of approach has been used to generate *Hp*-specific T cell clones from the gastric antrum (D'Elios et al., 2003). However $CD4^+$ T cells are known to have some plasticity and culture conditions can affect their differentiation (Veldhoen et al., 2009). Many more cytokine positive event are generated with PMA/ionomycin stimulation, as used by Satoh *et al.* and Serelli-Lee *et al.* (Satoh et al., Serelli-Lee et al., 2012) but in view of the wide variety of T cell specificities in the peripheral blood antigen stimulation was chosen for this study.

Some $CD4^+IFN\gamma^+IL17^+$ cells were identified and there was a trend for these "double positive" cells to be more common in the *Hp*-infected group. Despite

this there was no significant correlation between $CD4^+IFN\gamma^+$ and $CD4^+IL\text{-}17^+$ cells. There was a trend towards a correlation between *IL17* and *IFNG* expression in gastric tissue, as measured in independent RT-qPCR experiments (r=0.39, p=0.051) (Chapter 5).

IL-17 and IFNγ co-producing cells have been described, at sites of tissue inflammation (Annunziato et al., 2007, Ivanov et al., 2006, Kebir et al., 2009, Brucklacher-Waldert et al., 2009b) and in blood (Kebir et al., 2009, Brucklacher-Waldert et al., 2009b). Th17 cells can switch their phenotype to produce IFNγ *in vitro* (Shi et al., 2008, Lee et al., 2009) and *in vivo* (Bending et al., 2009, Shi et al., 2008, Lee et al., 2009). These double producers seem to be more like Th17 cells than Th1 cells in terms of the range of cytokines they produce and can upregulate defensin expression in epidermal keratinocytes (Boniface et al., 2010). They express the genes for both RORγt and T-bet transcription factors (Boniface et al., 2010, Abromson-Leeman et al., 2009, Zielinski et al., 2012), though t-bet has been shown to be able to inhibit RORγt transcription by sequestering Runx1 (Lazarevic et al., 2011). Fate mapping studies in mice engineered to express a yellow fluorescent protein in cells that had ever activated the IL-17 program indicate that $CD4^+IFN\gamma^+IL\text{-}17^+$ cells are derived from Th17 cells and IL-23 is required for upregulation of T-bet and IFNγ secretion by Th17 cells (Hirota et al.). IL-23 was required for terminal Th17 differentiation and for Th17 cells to have full pathogenic function in EAE and a model of skin inflammation (McGeachy et al., 2007, McGeachy et al., 2009). When the Th17 cells start to produce IFNγ they downregulate IL-23 receptor and CCR6 expression but retain AhR and IL-1R1 expression (Hirota et al.). This type of cell is at the more pro-inflammatory end of the Th17 spectrum (Peters et al., 2011).

Th17 cells may respond to different pathogens with different cytokines. Zielinkski *et al.* found that human naive $CD4^+$ T cells incubated with autologous monocytes pulsed with *C. albicans* developed a substantial $IL\text{-}17^+IFN\gamma^+$ population, whereas naive $CD4^+$ T cells incubated with

Staphylococcus aureus pulsed monocytes developed very few IL-17^{+}IFNγ^{+} cells. After restimulation some *S. aureus*-specific, but not *C. albicans*-specific Th17 clones produced IL-10 (Zielinski et al., 2012). In this system IL-1β was crucial for development of CD4^{+}IFNγ^{+}IL-17^{+} cells (Zielinski et al., 2012). In contrast, when mice were cutaneously infected with *C. albicans* hardly any of the IFNγ produced by CD4^{+} T cells originated from Th17 cells (Hirota et al.).

T-bet expressing CD4^{+}IL-17^{-}IFNγ^{+} populations differentiated under Th17 inducing (IL-1β, IL-23, and PGE2) conditions *in vitro* (Boniface et al., 2010) or derived from Th17 cells (Hirota et al., Lee et al., 2009) have been described, which have characteristics more like the Th17 than Th1 subset, suggesting additional phenotyping may be required to confirm the identity of Th1 cells.

4.4.3 The Peripheral Blood Treg Response to *Hp*

In this study only the CD4^{+}CD25^{high} Tregs were shown to be significantly increased in peripheral blood of *Hp*-infected patients compared to uninfected controls, although there were trends for increased CD4^{+}CD25^{high}CD127^{lo} and CD4^{+}CD25^{high}FOXP3^{+} Tregs. This is consistent with the findings of other groups (Satoh et al.) and FOXP3 real-time qPCR data from the research group (Greenaway et al., 2011). Lundgren *et al.* found increased CD4^{+}CD25^{high} Tregs in the stomach but not the blood of *Hp*-infected patients (Lundgren et al., 2005) but the numbers in their study were small. It might be expected that difference would be more marked at the site of infection itself compared to the blood. As CD25 is also a marker of activation it could be argued that these results simply reflect more activated CD4^{+} T cells in the peripheral blood of *Hp*-infected patients upon stimulation with *Hp* antigen, as shown earlier in this chapter with the activation marker CD69. However, CD25^{high} has been shown to be a specific feature of Tregs (Baecher-Allan et al., 2005) and as discussed in section 1.4.2.5 there are different subgroups of Tregs that do not all express FOXP3, such as IL-10 secreting Tr1 cells. This was a relatively small study with only 14 patients each in the *Hp*-infected and uninfected groups and doesn't exclude the possibility that increases in FOXP3 and CD127^{lo} might

be found in the peripheral blood of *Hp*+ patients in a larger study. These results in combination with others from my colleagues demonstrating increased $CD4^+CD25^{high}IL\text{-}10^+$ and $CD4^+CD25^{high}CTLA\text{-}4^+$ T cells and increased IL-10 by real-time qPCR in peripheral blood (Greenaway et al., 2011) make a compelling case that the increase in Tregs in the peripheral blood of *Hp*-infected patients is real.

Miyara *et al.* describe three subpopulations of human $FOXP3^+$ $CD4^+$ T cells. "Fraction I" is $FOXP3^{lo}CD45RA^+CD25^{++}$ and thought to represent resting Treg (rTreg) cells. "Fraction II" is $FOXP3^+CD45RA^-CD25^{+++}$, has the highest CTLA-4 expression and highest proliferation index, and is thought to represent activated Tregs (aTregs). Both these populations were suppressive *in vitro. In vivo* experiments indicate that rTregs can convert to aTregs. Microarray analysis found more IL-10 transcription in aTregs but more TGF-β expression in rTregs. The third population with a $FOXP3^{lo}CD45RA^-CD25^{++}$ phenotype (fraction III) was the greatest producer of IL-17 and did not exhibit suppressive function (Miyara et al., 2009). The increased $CD4^+CD25^{high}IL\text{-}10^+$ and $CD4^+CD25^{high}CTLA\text{-}4^+$ T cells reported in the peripheral blood of *Hp*-infected patients could fit with a "fraction II" aTreg phenotype though no increase in $CD4^+FOXP3^+$ cells in the peripheral blood of *Hp*-infected patients has been detected to date.

Whether the peripheral blood response reflects a "spill over" of the local immune response or reflects separate populations of cells, which might perhaps home to or from the stomach is unclear. Strong gastric Treg responses to *Hp* are associated with protection from peptic ulcer disease (Robinson et al., 2008). This study did not find an association between peripheral blood $CD4^+CD25^{high}$ Tregs and peptic ulcer disease but my colleagues have shown associations between peptic ulcer disease and lower frequencies of $CTLA\text{-}4^+$ and $IL\text{-}10^+$ $CD4^+CD25^{high}$ peripheral blood Tregs (Greenaway et al., 2011), reflecting the findings in the stomach. It is possible that the $CD4^+CD25^{high}$ cell population contains some other activated cells in

addition to the Tregs and more specific markers for Tregs induced by *Hp* are required.

In a large study by Wang *et al.* peripheral blood parameters in patients with *cag+* or *cag- Hp* and uninfected controls with pathology at different stages along the Correa pathway were measured. As the pathology progressed the Th1/Th2 ratio reduced and the Treg levels increased in those infected with *cag+ Hp* strains, suggesting gastric pathology is reflected at some level in the blood (Wang et al., 2007b).

On the other hand although there was no significant difference in frequencies of FOXP3 or $CD127^{lo}$ $CD4^{+}CD25^{high}$ events between *Hp*-infected and uninfected patients in blood, FOXP3 expression is increased in *Hp*-infected stomach and correlates with TGF-β1 expression (Kandulski et al., 2008, Hussain, 2012). This suggests that gastric and peripheral blood Tregs may have distinct phenotypes.

The increase in Tregs in peripheral blood of *Hp*-infected patients raises the possibility that *Hp* could have wider effects on the host immune response, distant to the stomach. Epidemiological studies have shown an inverse association between *Hp* infection and asthma and allergy, particularly infection with *cag+ Hp* strains (Chen and Blaser, 2007, Amberbir et al., 2011), reviewed by Blaser *et al.* (Blaser et al., 2008). *Hp* infection protects against asthma and allergy in mouse models (Arnold et al., 2011a). DC-derived IL-18 induced Treg development following exposure to *Hp* in this model (Oertli et al., 2012).

Epidemiological studies also show inverse associations between *Hp* infection and autoimmune conditions such as multiple sclerosis (Li et al., 2007). Conversely *Hp* eradication is advocated as a treatment for the autoimmune disease immune thrombocytopaenic purpura (Malfertheiner et al., 2012, Stasi et al., 2009, Fock et al., 2009).

Hp infection is common, especially in those with lower socioeconomic status, so epidemiological studies need to be carefully controlled to account for possible confounding factors, including other infections.

Having demonstrated an *Hp*-specific Th17 response in the peripheral blood of infected patients the rest of this thesis will focus on the local immune response to *Hp* in the stomach. Data on cytokine levels in the *Hp*-infected gastric mucosa are presented in Chapter 5.

CHAPTER 5

IL-17 AND OTHER TH17-RELATED CYTOKINES IN THE GASTRIC MUCOSA

5. IL-17 AND OTHER TH17-RELATED CYTOKINES IN THE GASTRIC MUCOSA

5.1 INTRODUCTION

As Th17 is a relatively newly recognized T helper cell population, relatively little is known about IL-17 and other Th17-related cytokines in the context of *Hp* infection in humans. Human research has mainly focused on Th1 and regulatory T cell responses to *Hp*. Relative levels of Th1-related and Th17-related cytokines in human *Hp* infection have not previously been studied, and many investigators have measured cytokines after *in vitro* stimulation of gastric biopsies or cells, which can alter their cytokine profile, depending on culture conditions (Veldhoen et al., 2009). The importance of IL-17 in *Hp*-induced disease is also unclear. These questions will be addressed in this chapter by using Luminex to analyze cytokine concentrations in snap frozen biopsies, which should more accurately reflect the *in vivo* cytokine concentrations. First the existing knowledge about Th17-related cytokines, Th1-related cytokines and other cytokines known to have roles in *Hp* gastritis including IL-8, TNF-α, regulatory cytokines and Th2 cytokines are reviewed.

5.1.1 Cytokines Produced by Th17 Cells and Cytokines Involved in Th17 Differentiation.

5.1.1.1 IL-23, a key cytokine for terminal Th17 differentiation is found at increased concentrations in Hp infection

The discovery of IL-23 in 2000 (Oppmann et al., 2000) led to the discovery of Th17 cells (Aggarwal et al., 2003, Harrington et al., 2005, Park et al., 2005), as described in section 1.3.1. IL-23 was known to promote Th17 cells but was found not to be critical for the initial differentiation of human Th17 cells from naïve $CD4^+$ T cells (Bettelli et al., 2006, Veldhoen et al., 2006, Mangan et al., 2006). Levels of IL-23 receptor (IL-23R) are low on naïve $CD4^+$ T cells but are upregulated upon activation of these cells in the presence of TGF-β and proinflammatory cytokines (Mangan et al., 2006, McGeachy et al., 2009). Studies using bone marrow chimeric mice with some IL-23R deficient and

some normal cells indicate that IL-23 is required for terminal differentiation and Th17 effector function *in vivo* (McGeachy et al., 2009). This is consistent with the protection of IL-23p19 subunit deficient mice from models of autoimmune disease (Cua et al., 2003, Murphy et al., 2003) and the finding that IL-23R polymorphisms are associated with inflammatory bowel disease and psoriasis (Duerr et al., 2006, Dubinsky et al., 2007, Cargill et al., 2007).

Hp can induce DCs to secrete IL-23 (Chapter 3, (Khamri et al., 2010)). Caruso *et al.* found increased IL-23 in *Hp+* human gastric biopsies by ELISA and increased IL-23p19 in the biopsies by RT-qPCR (Caruso et al., 2008). Gastric lamina propria mononuclear cells produced IL-17 in response to IL-23 *in vitro* and this could be abrogated by anti-IL-23 antibody or a STAT3 inhibitor (Caruso et al., 2008). Serelli-Lee *et al.* also found increased IL-23p19 in *Hp+* gastric biopsies, though the differences between the groups were small (Serelli-Lee et al., 2012). It has been reported that serum IL-23 levels are elevated in *Hp*-infected patients (Jafarzadeh et al., 2009). IL-23 can also drive IL-17 production by innate lymphoid cells (Buonocore et al., 2010).

5.1.1.2 IL-17: Effects and concentrations in the Hp- *infected gastric mucosa*

IL-17 itself is recognized by a heterodimeric receptor made up of IL-17RA and IL-17RC. This receptor can also recognize IL-17F and IL-17A/F heterodimers and is present on epithelial, endothelial and stromal cells.

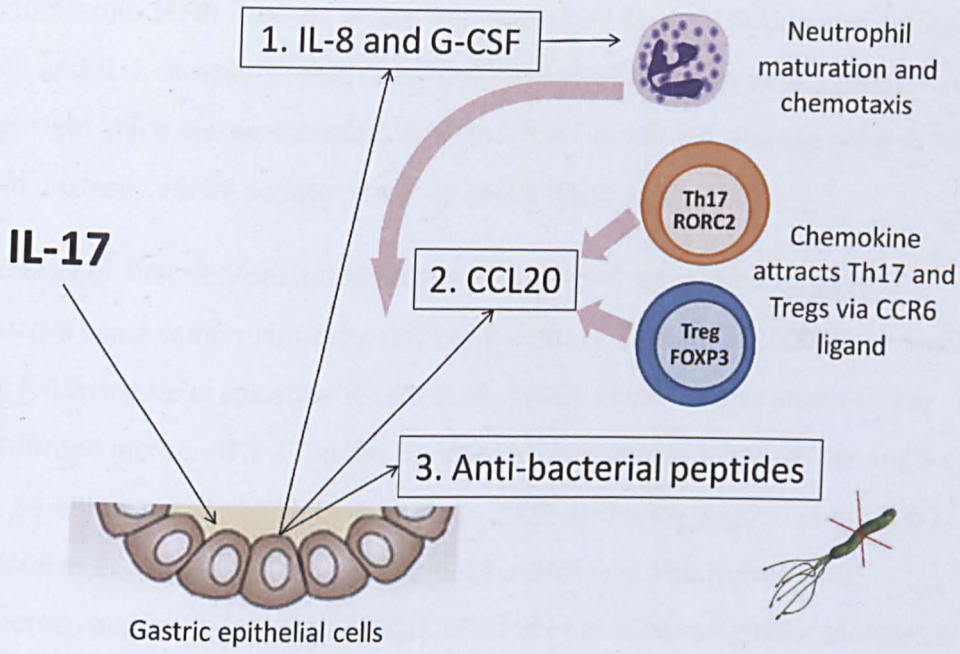


Figure 4.1 Effects of IL-17 mediated via gastric epithelial cells include induction of IL-8, G-CSF, CCL20 and anti-bacterial peptide genes.

Genes that IL-17 induces in these cells include anti-microbial proteins (including β-defensins, calgranins, cathelicidin and lipocalin-2), chemokines and neutrophil-activating factors (including G-CSF, GM-CSF, CXCL1, CXCL5, IL-8 and CCL20), acute phase proteins (including IL-6 and SAA) and matrix metalloproteinases (MMPs) (Gaffen, 2008, Gaffen, 2011) (Figure 4.1). CCR6, the CCL20 ligand is present on Th17 cells and Tregs. CCL20 is increased in the *Hp*-infected stomach and falls following *Hp* eradication (Yoshida et al., 2009, Serelli-Lee et al., 2012, Wu et al., 2007).

Extracellular signal-regulated protein kinase 1/2 (ERK1/2), AP-1, NF-κB and IL-8 synthesis were induced by IL-17 treatment of the MKN28 gastric epithelial cell line and gastric epithelial cells from *Hp*-infected patients had higher ERK1/2 activation and IL-8 levels, particularly the CagA seropositive subset (Luzza et al., 2000). Act1 is the key signaling molecule bound by the IL-17 receptor to trigger downstream signaling pathways including MAPK and NF-κB (Chang et al., 2006, Qian et al., 2007, Ho et al.) . The IL-17 receptor has a

cytoplasmic SEFIR domain, which has homology to the TIR domains found in Toll and IL-1 receptors which can bind Act1 (Novatchkova et al., 2003). Act1-deficient mice were protected from the IL-17-mediated disease models EAE and dextran sodium sulfate –induced colitis (Qian et al., 2007).

Luzza *et al.* first demonstrated increased IL-17 in the *Hp*-infected gastric mucosa using semi-quantitative PCR and western blotting in 2000. IL-17 levels fell following *Hp* eradication (Luzza et al., 2000). Several other studies have confirmed increased IL-17 in the *Hp*-infected stomach in humans (Kimang'a et al., Serelli-Lee et al., 2012, Caruso et al., 2008) and mice (Algood et al., 2007, Shiomi et al., 2008, Shi et al., 2010). IL-17 mRNA was also higher in *Hp* infected children (Luzza et al., 2001). Mizuno *et al.* cultured gastric biopsies in media with phytohaemagluttinin for 48 hours and measured IL-17 and IL-8 levels by ELISA in the supernatants. Both IL-17 and IL-8 were increased in the supernatants of *Hp*-infected compared to uninfected biopsies and IL-17 levels correlated with IL-8 levels and neutrophil infiltration. No difference was found in cytokine levels between *Hp*+ patients with or without peptic ulcer disease, though supernatants from ulcer sites contained more IL-17 than those from the antrum of the same patient (Mizuno et al., 2005).

5.1.1.3 Concentrations of IL-17 target chemokines in Hp *infection*

CCL20 and β-defensin-2 gene expression are increased in gastric biopsies from *Hp*+ patients compared to those from uninfected patients but correlations with IL-17 levels have not been investigated (Wu et al., 2007, Serelli-Lee et al., 2012, Yoshida et al., 2009). IL-8 and GROα (also known as CXCL1 and KC) chemokines, also IL-17 targets, were increased in *Hp* infected gastric biopsies and correlated with *Hp* density and neutrophil and lymphocyte infiltration (Yamaoka et al., 1998). A number of studies have shown IL-8 secretion in response to IL-17 in human gastric epithelial cells lines (Luzza et al., 2000, Mizuno et al., 2005, Sebkova et al., 2004). Mouse gastric epithelial cells and fibroblasts produced the chemokines KC (CXCL1) and LIX (CXCL5) in response to recombinant IL-17 (DeLyria et al., 2009). IL-17 increased expression of

MMP-2, MMP- 3, MMP-7, MMP- 9, CCL2, CCL5, CCL20, CCL25, CXCL1 in a mouse gastric cell line (Shi et al., 2010). IL-17RA knockout mice expressed lower levels of KC, LIX and GM-CSF and in keeping with this had lower neutrophil infiltration and higher *Hp* colonization than their wild-type counterparts (Algood et al., 2009). Gastric IL-17 and IL-21 mRNA levels and lymphocyte infiltration were increased in the IL-17RA knockout animals and they also had increased gastric B cells, lymphoid follicles and *Hp*-specific antibody responses, leading the authors to propose that IL-17 signaling has a role in regulation of B cell recruitment (Algood et al., 2009).

5.1.1.4 IL-17 responses in mouse models of Hp *infection*

IL-17 responses are associated with protection against *Hp* infection in mouse vaccination models. DeLyria *et al.* found increased gastric IL-17 mRNA in immunized compared to unimmunized mice by day 3 post-challenge. Protection was abrogated in neutrophil-depleted immunized mice (DeLyria et al., 2009). Velin *et al.* found increased gastric IL-17 mRNA and protein on day 10 after *H. felis* challenge in another mouse vaccination model. Vaccine-induced protection against *Helicobacter* was abrogated by anti-IL-17 antibody treatment (Velin et al., 2009, Flach et al.). Kao *et al.* adoptively transferred *Hp*-pulsed BMDCs, then infected the mice with *Hp* in a study to investigate the balance of Treg and Th17 differentiation. In this study the IL-17 was inversely correlated with *Hp* colonization density at 2 weeks, but this correlation did not persist to the 6 week time point (Kao et al., 2010). However in a further study by DeLyria *et al.*, immunized IL-17 and IL-17RA knockout mice were not compromised in their ability to reduce *Hp* load, even when anti-IFNγ was also given, suggesting substantial redundancy in the immune response to *Hp* vaccination (DeLyria et al., 2011).

Studies with IL-17 knockout mice have shown reduced neutrophil infiltration and reduced *Hp* colonization compared to wild-type (Shiomi et al., 2008, Shi et al., 2010, DeLyria et al., 2011). This suggests that IL-17 is not protective in these models. However IL-17RA knockout mice had increased levels of colonization in addition to reduced neutrophil infiltration, suggesting that IL-

17 signalling can mediate protection (Algood et al., 2009). Shi *et al.* found IL-17 increased before IFNγ in response to *Hp* infection and IL-17 knockout mice also had reduced Th1 responses (Shi et al., 2010). These findings suggest that neutralizing IL-17 may be a good therapeutic strategy to reduce colonization levels and *Hp*-associated pathology.

A study in which C57BL/6J mice were treated with anti-IL-17A 6 months after *Hp* infection suggested that IL-17 may have an anti-inflammatory role in *Hp*-induced gastritis. Levels of gastric IFNγ and TNFα mRNA were increased in the mice treated with anti-IL-17A, though IL-12 levels were unchanged (Otani et al., 2009).

5.1.1.5 Th17 effector cytokines in Hp *infection*

$CD4^+$ Th17 cells are a major source of IL-17 in chronic inflammation and infection (sections 1.3.4 and 1.3.5) but are not the only source of this cytokine. Other sources include $CD8^+$ T cells, γδ T cells, NKT cells, macrophages, LTis and other ILC3s, paneth cells, and under some circumstances neutrophils (see section 1.3.9)(Gaffen, 2011, Cua and Tato, Takatori et al., 2009, Takahashi et al., 2008, Buonocore et al., 2010).

IL-17F can also be produced by Th17 cells and signal through the IL-17RA/C receptor. IL-17F was less potent than IL-17 at inducing IL-8 expression in gastric epithelial cell lines (Zhou et al., 2007b). In a study using gene knockout mice Ishigame *et al.* showed that IL-17F had little role in mouse models of autoimmune disease but worked together with IL-17 in defence against mucosal infections, particularly by activating epithelial cell innate immunity (Ishigame et al., 2009). Data on this cytokine in the context of *Hp* infection is limited. Kimang'a *et al.* studied cytokine expression in gastric biopsies of subjects from Kenya and Germany by RT-PCR and found increased IL-17F expression in *Hp* infection. This correlated with IL-17 expression (Kimang'a et al.).

IL-22 is also produced by Th17 cells. It is a member of the IL-10 family and mediates its effects through the IL-22R which is expressed on epithelial cells

and pairs with IL-10R2. Like IL-17, IL-22 can induce expression of proinflammmatory cytokines, chemokines and acute phase proteins and also induces expression of antimicrobial peptides including β-defensins and S100-family proteins (Ouyang et al., 2008). It can work with IL-17, for example to increase defensin expression in keratinocytes (Liang et al., 2006). It has a crucial role in defence against *K. pneumonia* and *C. rodentium* in mice (Aujla et al., 2008, Satoh-Takayama et al., 2008). In addition to its roles in inflammation and host defense IL-22 affords some protection against tissue damage (Zenewicz et al., 2007) and induces genes involved in wound healing and tissue repair (Sa et al., 2007). IL-22 is increased in inflamed skin and Eyerich *et al.* derived T cell clones that secrete IL-22 but not IL-17 from skin biopsies, leading to the proposal of a Th22 lineage (Eyerich et al., 2009). Though it has probably been best studied in the skin IL-22 is also increased in inflammation associated with rheumatoid arthritis and inflammatory bowel disease (Wolk et al.).

Data on IL-22 in *Hp* infection are limited. Obonyo found that wild-type mice had higher IL-22 expression than MyD88-deficient mice following infection with *H. felis* (Obonyo et al., 2011). Serelli-Lee *et al.* found no significant difference in IL-22 expression in gastric biopsies from uninfected patients and those with current or previous *Hp* infection by immunofluorescence microscopy or ELISA (Serelli-Lee et al., 2012).

T cells are a major source of IL-22 but it can also be produced by ILC3s including LTi and NCR^+ cells in the intestine (Cupedo et al., 2009, Wolk et al., Satoh-Takayama et al., 2008). Murine intestinal $p46^+$ NK cells (NCR^+ ILC3s) differ from conventional NK cells in that they have low expression of genes for cytotoxic molecules and IFNγ (Satoh-Takayama et al., 2008). They express RORγt but not IL-17 (Satoh-Takayama et al., 2008). Using lymph nodes from human fetal tissue Cupedo *et al.* showed that $CD4^-$ LTi cells could differentiate into $CD56^+CD127^+RORC^+$ NK cells that produced IL-17 and IL-22 but not IFNγ (Cupedo et al., 2009).

5.1.1.6 IL-21 in Hp infection

IL-21 can be produced by Th17 cells and can also act in an autocrine manner to promote their differentiation (Nurieva et al., 2007, Zhou et al., 2007a, Korn et al., 2007). Like IL-6, IL-21 can act via STAT3 to promote Th17 development at the expense of Treg development in pro-inflammatory environments (Nurieva et al., 2007, Zhou et al., 2007a). IL-21R forms a receptor complex with the common gamma chain and is expressed on T cells, B cells, NK cells, dendritic cells, macrophages, fibroblasts and epithelial cells, including gastric epithelial cells (Ouyang et al., 2008, Caruso et al., 2007a, Monteleone et al., 2006) so IL-21 can have a broad range of effects. IL-21 promotes IgG and suppresses IgE synthesis by B cells. Germinal centre T follicular helper cells produce abundant IL-21 which is crucial for germinal centre formation (Spolski and Leonard). IL-21 also has roles in maturation and stimulation of IFNγ secretion from Th1, $CD8^+$ T cell and NK cells (Ouyang et al., 2008). Brandt *et al* found that IL-21 inhibited the activation and maturation of murine BMDCs (Brandt et al., 2003)

IL-21 is produced by activated T cells and increased levels have been shown in biopsies from *Hp* infected patients (Caruso et al., 2007a), as well as biopsies from patients with other gastrointestinal inflammatory diseases including Crohn's disease, ulcerative colitis and coeliac disease (Monteleone et al., 2005, Fantini et al., 2008, Fina et al., 2008). IL-21 increased secretion of the chemokine CCL20 by a colon epithelial cell line (Caruso et al., 2007b). It also promotes tissue damage by increasing expression of matrix metalloproteinases (MMPs), as demonstrated using gastric epithelial cells lines and fibroblast cell lines derived from the colons of patients with Crohn's disease (Caruso et al., 2007a, Monteleone et al., 2006).

Algood *et al* infected IL-17RA knockout and wild-type mice with *Hp* and measured Th17 cytokine production by RT-qPCR at 3 and 6 months. Levels of IL-21 were increased at both time points in the IL-17RA knockout mice, as were IL-17 levels. The authors suggest that this could be due to lack of a negative feedback loop (Algood et al., 2009).

5.1.1.7 Other Th17-differentiating cytokines in Hp *infection: IL-1β and IL-6*

IL-1β is involved in differentiation of human Th17 cells (Acosta-Rodriguez et al., 2007a, Volpe et al., 2008) but was of interest in *Hp* research before the discovery of Th17 cells. It stimulates secretion of the hormone gastrin (Levi et al., 1989, Robinson et al., 2007, Takashima et al., 2001), which in turn stimulates gastric acid secretion. However, IL-1β itself inhibits gastric acid secretion in animal models (Takashima et al., 2001, Uehara et al., 1989, Waghray et al.). IL-1β polymorphisms are associated with gastric cancer risk (El-Omar et al., 2000). Sonic hedgehog, a morphogen initially identified in Drosophila, may contribute to the link between IL-1β and cancer as IL-1β inhibited sonic hedgehog expression (Waghray et al.) and reduced sonic hedgehog expression is associated with precancerous changes (Shiotani et al., 2005). Gastric IL-1β is upregulated in infection by *cagA+ Hp* strains (Yamaoka et al., 1997). IL-1β increases IL-8 and MMP-3 expression in gastric epithelial cell lines (Gooz et al., 2003). Eradication of *Hp* does not always prevent the development of gastric cancer, particularly if precancerous changes have begun to develop (Wong et al., 2004). Serelli-Lee *et al.* found that gastric IL-1β levels remained elevated in patients who had previously been infected with *Hp*. They hypothesize that this may drive the persistent Th17 response they identify following *Hp* eradication and that chronic Th17 gene activation may lead to carcinogenesis (Serelli-Lee et al., 2012). Active IL-1β is cleaved from pro-IL-1β precursor by caspase-1. IL-1R$^{-/-}$ mice failed to develop vaccine-induced immunity to *Hp* but were protected against gastric inflammation and precancerous changes (Hitzler et al., 2012b). IL-1β is produced by antigen presenting cells including macrophages and dendritic cells (Chapter 3) and also by gastric epithelial cells (Lindholm et al., 1998).

Like IL-1β, the proinflammatory cytokine IL-6 is involved in Th17 differentiation (Bettelli et al., 2006, Acosta-Rodriguez et al., 2007a, Manel et al., 2008, Zhou et al., 2007a). Increased levels of IL-6 are present in *Hp*-infected gastric mucosa (Crabtree et al., 1991). *Hp* induces IL-6 production by

the gastric epithelium (Lindholm et al., 1998) as well as strongly upregulating IL-6 secretion by antigen presenting cells (Chapter 3), (Odenbreit et al., 2006, Gobert et al., 2004). IL-6 is increased in gastric cancer but, unlike IL-8, fell with *Hp* eradication (Yamaoka et al., 2001).

5.1.2 Cytokines Produced by Th1 Cells and Cytokines Involved in Th1 Differentiation

5.1.2.1 IFNγ response to Hp *infection in humans*

Early studies found increased IFNγ$^+$ in the *Hp*-infected stomach that correlated with inflammation using immunohistochemistry (Lindholm et al., 1998, Lehmann et al., 2002, Holck et al., 2003). Flow cytometry studies in the late 1990s found increased IFNγ$^+$ lymphocytes in *Hp*-infected human gastric biopsies following *in vitro* stimulation with PMA and ionomycin (Bamford et al., 1998, Sommer et al., 1998) but gastric T cells from uninfected patients can also secrete IFNγ following mitogen stimulation (Sommer et al., 1998, Itoh et al., 1999). More recent flow cytometry data and studies looking at cytokine production by cells cloned from *Hp*-infected gastric mucosa are discussed in section 1.4.2.3. Lehmann *et al.* found increased IFNγ and TNF-α staining in gastric biopsies from *Hp*-infected patients which positively correlated with activity and grade of gastritis, local density of *Hp* and apoptosis of stromal cells (Lehmann et al., 2002). Bontems *et al.* found increased IFNγ in *Hp*-infected biopsies compared to uninfected biopsies from both adults and children with approximately 10-fold lower concentration in children compared to adults using cytokine bead array, adjusted for biopsy weight and ELISPOT (Bontems et al., 2003). Shimuzu *et al.* also studied gastric biopsies from children but homogenized in PBS with ultrasound homogenizer and adjusted ELISA results for total protein. They confirmed higher IFNγ levels in antral biopsies of *Hp*-infected compared to uninfected children but did not find any correlation with neutrophil or lymphocyte infiltration (Shimizu et al., 2004).

Pellicano *et al.* disrupted biopsies using a lysis buffer and found increased IFNγ and IL-12 in *Hp*-infected compared to uninfected biopsies by ELISA (adjusted for total protein). Those with higher mononuclear cell infiltrates had higher IL-12 and IFNγ levels (Pellicano et al., 2007).

Case-control studies indicate that a polymorphism in the interferon gamma receptor 1 is associated with increased risk of early onset gastric cancer (Canedo et al., 2008).

5.1.2.2 IFNγ response to Hp *in mice and Mongolian gerbils*

Mice also mount an IFNγ response to *Hp* infection (Sawai et al., 1999, Smythies et al., 2000, Algood et al., 2007, Akhiani et al., 2002). IFNγ knockout mice were protected from gastric inflammation (Smythies et al., 2000, Akhiani et al., 2002, Sawai et al., 1999) and were not able to upregulate the inflammatory mediators inducible nitric oxide synthase (iNOS) and macrophage inflammatory protein-2 or clear *H. felis* infection like their wild-type counterparts (Obonyo et al., 2002, Hitzler et al., 2012b). Addition of IFNγ with *Hp* increased iNOS expression in gastric epithelial cells compared to treatment of the cells with *Hp* alone (Perfetto et al., 2004). A study using *Hp*-infected Mongolian gerbils found that IFNγ levels correlated with activity and inflammation and were higher in the gerbils with ulcers (Yamaoka et al., 2005). Sayi *et al.* found that in addition to correlating with chronic inflammation IFNγ levels correlated with the preneoplastic changes of atrophy and epithelial hyperplasia (Sayi et al., 2009). Even in the absence of *Hp* infection mice infused with IFNγ developed atrophy and intestinal metaplasia (Cui et al., 2003).

A number of studies report a protective role for IFNγ in mouse vaccination models (Akhiani et al., 2002, Sayi et al., 2009, Shi et al., 2005) but others found a small or no protective effect associated with IFNγ (Sawai et al., 1999, Garhart et al., 2003). In some vaccination studies IFNγ levels correlated with reduced colonization levels but there was no reduction in protection when

IFNγ was neutralized with antibody or IFNγ knockout mice were used (Flach et al., Garhart et al., 2003).

A recent study comparing wild type, p35 and p19 knockout mice infected with *H. felis* or *H. pylori* found no difference in colonization density between the three groups, suggesting that neither IL-12 nor IL-23 is essential for a protective response. The p19 knockout group did have some reduction in gastritis and preneoplasia in the *Hp* infection model (Hitzler et al., 2012a).

IFNγ is sometimes thought of as a T cell cytokine but can also be produced by NK cells in response to *Hp* (Lindgren et al.). THY1hiSCA1+ ILC3s also produce IFNγ and IL-17 in response to IL-23 (Buonocore et al., 2010).

5.1.2.3 IL-18 in Hp infection

IL-18 was identified as an IFNγ-inducing factor (Okamura et al., 1995) and is often described as a Th1 cytokine. Like IL-1β it is processed to its mature form by caspase-1. Tomita *et al.* found increased IL-18 expression in the antrum of *Hp*-infected patients compared to uninfected controls with normal mucosa using semi-quantitative PCR (Tomita et al., 2001). Yamauchi *et al.* also found increased IL-18 in the *Hp*-infected gastric antrum at both the mRNA and protein level and demonstrated IL-18 secretion by primary gastric epithelial cells in response to *Hp*. In contrast, Kimang'a *et al.* found that IL-18 mRNA levels did not differ between *Hp+* and *Hp-* patients from Germany and Kenya (Kimang'a et al.). Some of the uninfected patients in this group did have a degree of inflammation on histopathology scoring whereas the *Hp*-infected patients were compared to uninfected patients with normal mucosa in the other two studies. It is not clear which part of the stomach the biopsies used for the RT-qPCR studies were taken from in Kimang'a *et al.'s* study. More recently IL-18 has been shown to drive differentiation of Tregs which can prevent the development of asthma in a mouse model (Oertli et al., 2012). In keeping with this regulatory role for IL-18, IL-18$^{-/-}$ mice had increased inflammation and precancerous changes one month after *Hp* infection compared to their wild-type littermates (Hitzler et al., 2012b).

5.1.3 Other Cytokines Investigated in the *Hp*-Infected Gastric Mucosa

5.1.3.1 Other pro-inflammatory cytokines: IL-8 and TNF-α

IL-8 is a neutrophil chemoattractant. Increased IL-8 levels are found in the *Hp*-infected gastric mucosa and are associated with *Hp* density and inflammation scores (Yamaoka et al., 1998, Yamaoka et al., 1997, Holck et al., 2003, Kimang'a et al., Shimizu et al., 2004, Yamaoka et al., 1995). IL-8 is expressed by gastric epithelial cells via an NF-κB-dependent pathway (Sharma et al., 1998). Gastric epithelial cell lines express more IL-8 when exposed to *cag+ Hp* strains than *cag-* strains (Sharma et al., 1995). Increased IL-8 is also found in gastric biopsies of patients infected with *cag+* strains (Yamaoka et al., 1998, Yamaoka et al., 1997). *In vitro* studies using cell lines suggest that CagA is necessary for epithelial TLR2 and TLR5 upregulation which allows IL-8 and TNF-α expression (Kumar Pachathundikandi et al.). Usually IL-8 falls following *Hp* eradication (Moss et al., 1994, Serelli-Lee et al., 2012), but levels are increased in gastric cancer, especially advanced stage, where they appear to be independent of *Hp* infection (Yamaoka et al., 2001). As mentioned above, IL-8 is now known to be an IL-17 target gene.

Increased TNF-α has been found in *Hp*-infected gastric mucosa using a range of methods (Crabtree et al., 1991, Lindholm et al., 1998, D'Elios et al., 1997, Bodger et al., 1997). It was higher in *Hp*-infected patients with active gastritis (Crabtree et al., 1991) and correlated with *Hp* density and inflammation scores (Lehmann et al., 2002). However, Bontems *et al.* reported that there was no difference in numbers of TNF-α secreting cells between *Hp*-infected and uninfected adults or children (Bontems et al., 2003). This was a relatively small study. EliSPOT was used to investigate the number of cytokine-secreting cells whereas the total amount of cytokine secreted or mRNA levels were measured in the other studies. Yamaoka *et al.* did not detect any TNF-α mRNA at all in *Hp*-infected or uninfected patients (Yamaoka et al., 1995), but later reported increased TNF-α in *Hp*-infected antral biopsies at the protein

level (Yamaoka et al., 1997). TNF-α and IL-1β upregulate Fas *in vitro* (Houghton et al., 2000), and Fas-induced apoptosis is thought to contribute to breakdown of the epithelial barrier in *Hp* infection (Suerbaum and Michetti, 2002). Proinflammatory polymorphisms in TNF-α increase gastric cancer risk (El-Omar et al., 2003). Two proteins produced by *Hp* that can induce TNF-α secretion and translocate into host cells have been identified: *HP0596* gene produces a protein known as TNF-α-inducing protein (Tipα) (Suganuma et al., 2005) and the *JHP940* locus produces a kinase (Rizwan et al., 2008, Kim et al.). *Hp* strains isolated from gastric cancer patients produced more Tipα than those from *Hp* patients with chronic gastritis (Suganuma et al., 2008).

5.1.3.2 Anti-inflammatory cytokines: IL-10 and TGF-β

Anti-inflammatory cytokines found in the gastric mucosa include IL-10 and TGF-β. Expression of both these cytokines is increased in the *Hp*-infected gastric mucosa (Li and Li, 2006, Robinson et al., 2008, Holck et al., 2003, Lindholm et al., 1998). Those with peptic ulcer disease had less gastric IL-10-secreting Tregs (Robinson et al., 2008). IL-27 can induce IL-10-secreting type 1 regulatory T (Tr1) cells at the expense of pro-inflammatory Th17 cells (Pot et al., 2011) and has been shown to induce aryl hydrocarbon receptor which can interact with c-maf, to promote T cell IL-10 secretion in the intestine (Apetoh et al., 2010). However, its expression in the *Hp*-infected stomach has not yet been studied. Bodger *et al.* found that IL-10 secretion of gastric biopsies from *Hp*-infected patients was significantly higher than that from gastric biopsies from uninfected patients with chronic gastritis (Bodger et al., 1997). *Hp*-infected gastric cancer patients produced more IL-10 upon *Hp* stimulation of their peripheral blood T cells and had higher gastric IL-10 mRNA levels than asymptomatic *Hp*+ patients (Lundin et al., 2007). Proinflammatory IL-10 polymorphisms are associated with an increased risk of non-cardia gastric cancer (El-Omar et al., 2003).

Hp increases TGF-β expression by the gastric epithelium and upregulates TGF-β receptor on $CD4^+$ T cells (Beswick et al., 2007). Li *et al.* found increased

mucosal TGF-β in *Hp* infection which correlated positively with the severity of gastritis (Li and Li, 2006). Similarly Kandulski found TGF-β correlated with FOXP3 expression, which also correlated positively with inflammation (Kandulski et al., 2008). Tregs are likely to be recruited to limit the pro-inflammatory response and may sustain inflammation by reducing anti-bacterial immunity. Robinson *et al's.* findings of low Treg frequencies in peptic ulcer disease (Robinson et al., 2008) suggests that the Tregs may protect from ulceration by reducing inflammation. Harris *et al.* found increased Tregs, IL-10 and TGF-β in *Hp*-infected children compared to adults, which could fit with the anti-inflammatory response protecting from *Hp*-induced disease (Harris et al., 2008).

5.1.3.3 IL-4

Hp-infected IL-$4^{-/-}$ mice had more severe gastritis than their wild-type counterparts, suggesting that IL-4 could also have an anti-inflammatory role (Smythies et al., 2000). This could be at least in part due to the mutual antagonism between IFNγ-producing Th1 cells and IL-4-producing Th2 cells. Studies investigating IL-4 expression at both the mRNA (in children) and protein level by immunohistochemistry reported no difference between *Hp*-infected and uninfected patients (Lindholm et al., 1998, Shimizu et al., 2004). Marotti *et al.* isolated mononuclear cells from gastric biopsies and counted the number of IL-4- secreting cells using EliSPOT. In keeping with the previously mentioned studies they found no difference between mononuclear cells from *Hp*-infected and uninfected patients when the cells were unstimulated, however, mononuclear cells isolated from gastric mucosa of *Hp*-infected patients showed increased numbers of IL-4-secreting cells upon *Hp* antigen stimulation whereas those from uninfected patients did not (Marotti et al., 2008). Robinson *et al.* also found a strong $CD45^{+}CD4^{+}IL\text{-}4^{+}$ response in *Hp*-stimulated mononuclear cells derived from *Hp*-infected biopsies compared to biopsies from uninfected patients using flow cytometry (Robinson et al., 2008). This study also found increased IL-4 mRNA in gastric biopsies from *Hp*-infected adults compared to uninfected controls (Robinson

et al., 2008). As discussed in Chapter 3, *Hp* promotes thymic stromal lymphopoietin production by gastric epithelial cells (Kido et al., 2010) which favours a Th2 bias (Rimoldi et al., 2005).

5.2 AIMS

i. To confirm that IL-17 levels are increased in the *Hp*-infected gastric mucosa.

ii. To determine the most sensitive method for quantifying IL-17 protein and other cytokines of interest in gastric biopsies and optimize this method.

iii. To compare relative levels of Th1 and Th17 cytokines.

iv. To correlate IL-17 levels with cag and dupA virulence factors.

v. To correlate IL-17 with histopathological findings and *Hp*-related disease.

5.3 RESULTS

5.3.1 IL-17 Levels are Increased in the *Hp*-Infected Gastric Mucosa

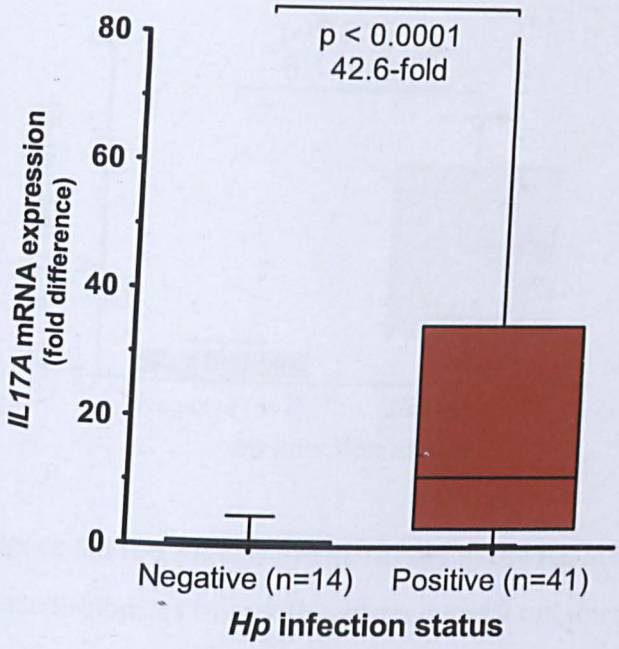


Figure 5.2 *IL-17A* mRNA levels increased in the *Hp*-infected gastric mucosa (RT-qPCR). mRNA was extracted from gastric tissue biopsies from 41 *Hp*-infected and 14 uninfected patients. Relative *IL-17A* levels were calculated by normalizing against *GAPDH* and expressing levels relative to a negative comparator reference sample prepared from 14 uninfected biopsies. Boxes represent the 25th to 75th centiles, horizontal lines within the boxes represent the median and the whiskers depict the 5th and 95th centiles. The Mann-Whitney U-test was used for Statistical analysis.

IL-17mRNA and protein expression was first investigated by RT-qPCR and ELISA to confirm increased IL-17 expression in the *Hp*-infected gastric mucosa.

IL-17 mRNA expression was 42.6-fold higher in biopsies from *Hp*-infected compared to uninfected patients ($p<0.0001$)(Figure 5.2).

To confirm these results at the protein level gastric biopsies were initially homogenized in 250 µl PBS and IL-17A ELISA performed on the supernatants. Results were adjusted for the total weight of gastric tissue.

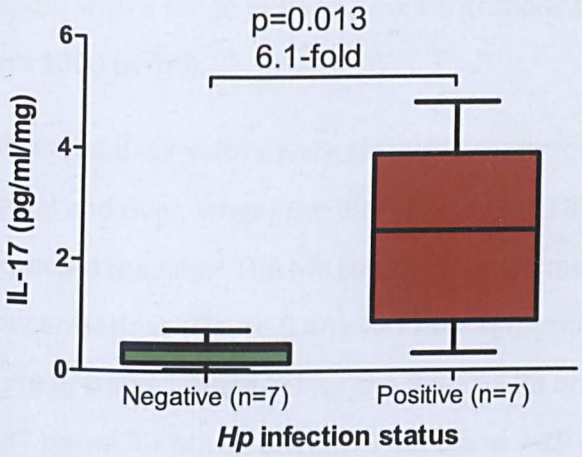


Figure 5.3 IL-17 levels are increased in the *Hp*-infected gastric mucosa. Gastric biopsies from 7 *Hp*-infected and 7 uninfected patients were homogenized in PBS and IL-17 measured by ELISA in the supernatants.

Multiple gastric biopsies were required for many of the samples from uninfected patients in order for IL-17 levels to reach sufficient concentration to be measured by ELISA.

Luminex technology enables measurement of multiple cytokines in a single sample of small volume (usually 25-50 µl compared to 100-200 µl per ELISA). This has the potential to allow quantitation of multiple cytokines in small gastric biopsies, without *in vitro* culture or stimulation, which may affect cytokine concentrations. Luminex kits from 3 different manufacturers were therefore compared and methods optimized.

5.3.2 Luminex Kit Comparison and Optimization

Luminex kits from 3 different manufacturers (R&D VersaMAP, BioRad BioPLEX and Millipore MILLIPLEX) were compared for accuracy, sensitivity and precision of IL-17 and IFNγ cytokine measurement. Biopsies were processed in RPMI-based media, as described in Material and Methods (Chapter 2) and spiked with a range of known concentrations of cytokine (0.75, 1.5, 6, 50, 100 and 1000 pg/ml).

Observed IL-17 values were closest to expected with the VersaMAP kit at 50 pg/ml and over, where the BioPLEX and MILLIPLEX kit gave lower than expected readings. The MILLIPLEX kit performed best at lower spiked concentrations (Figure 5.4A). IL-17 background levels in unspiked samples were 0, 0 and 1.78 pg/ml for the BioPLEX kit and a little higher at 0, 2.49 and 2.37 pg/ml for MILLIPLEX and 3.45, 0 and 7.19 pg/ml for the VersaMAP kits. Accuracy was poor below 6 pg/ml. For the 6, 50, 100 and 1000 pg/ml IL-17 spikes the percentage recovery ranged between 85-123% for VersaMAP, 31-47% for BioPLEX and 27-69% for MILLIPLEX. However, it could be argued that linearity and consistency are more important than the absolute values obtained.

The IFNγ spikes were recovered with the highest degree of accuracy in the MILLIPLEX kit. In general the VersaMAP kit gave lower than expected readings and the BioPLEX kit higher than expected readings for IFNγ (Figure 5.4B). High levels of background were detected in the wells containing supernatant from biopsies disrupted in RPMI/10%FCS/protease inhibitor with no cytokine spike with the BioPLEX kit when analyzing for IFNγ (49.19, 263.99 and 1193.72 pg/ml). Background levels with VersaMAP (2.25, 0.42 and 0 pg/ml) and MILLIPLEX (0.3, 6.73 and 4.54 pg/ml) kits were much lower. A well with RPMI/10% FCS/Protease inhibitor only yielded an IFNγ reading of 1177.74 pg/ml with the BioPLEX kit compared to 0 pg/ml for PBS/Protease inhibitor, suggesting that the IFNγ detection antibody in this kit may react with a component of the RPMI/10% FCS medium. Percentage recovery ranges for

IFNγ were 2.7-7.2% for VersaMAP, 57.5-476.0 for BioPLEX and 22.2-103.7 for MILLIPLEX.

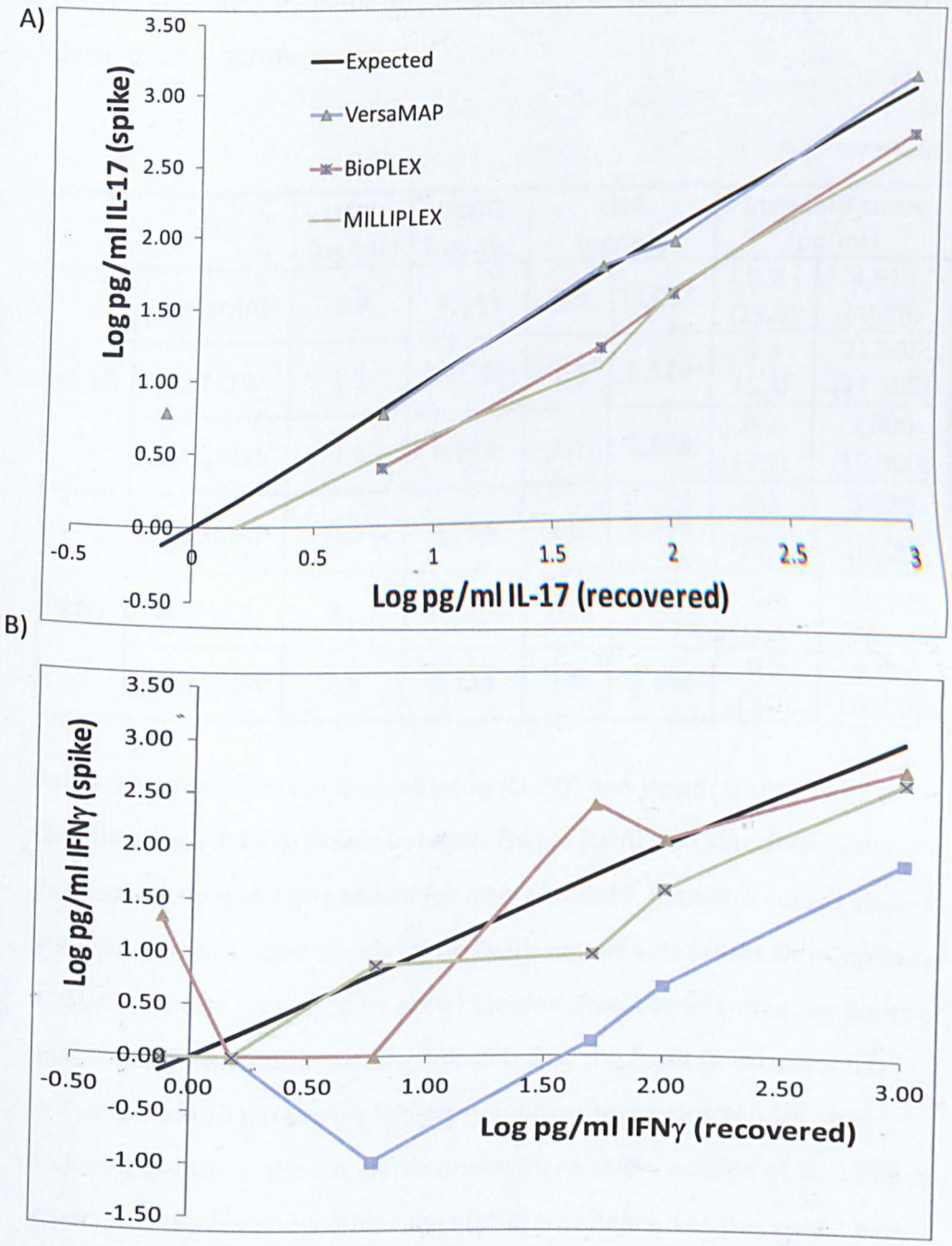


Figure 5.4 Recovery of spiked IL-17 (A) and IFNγ (B) with Luminex kits from 3 different manufacturers. Single biopsies were processed in RPMI-based media and the supernatants from 3 biopsies from the same donor combined. This supernatant was spiked with known concentrations of cytokine, then divided into 3 aliquots to test with the 3 different kits. Key for both shown at top left of A).

Next the sensitivity and operative range of each kit was compared by assessing the lower limit of quantitation (LLOQ), upper limit of quantitation (ULOQ) and linear dynamic range based on the standard curves extended down to ≤0.5 pg/ml.

		LLOQ (pg/mL)	ULOQ (pg/mL)	LDR (pg/mL)		Standard curve (pg/mL)	
IL-17	VersaMAP	5.9	4,643	5.9	4,643	0.2 (19.0)	4,616 (4616)
	BioPLEX	1.3	23,036	1.3	5,120	0.3 (1.3)	21,505 (21,505)
	MILLIPLEX	3.4	8,938	3.4	8,938	0.2 (3.2)	9,000 (10,000)
IFNγ	VersaMAP	0.3	5,754	1.0	2,398	0.3 (27.2)	6,620 (6620)
	BioPLEX	8.1	30,660	26.5	7,661	0.5 (1.9)	30,646 (30,646)
	MILLIPLEX	2.8	9,144	2.8	9,144	0.2 (3.2)	9,000 (10,000)

Table 5.1 Lower Limit of Quantitation (LLOQ) and Upper Limit of Quantitation (ULOQ), Linear Dynamic Range (LDR) and Standard Curve Ranges for the three kits tested for IFNγ and IL-17. Standard curves shown are based on extended dilution series with figures in brackets showing lower and upper limits suggested by each manufacturer. Lower and upper limits of quantitation were automatically calculated by the BioPLEX manager 6.0 software (using 5 parameter logistic non-linear regression model, after excluding points on the standard curve with recovery outside of 80-120% of expected). The linear dynamic range (LDR) was defined as the lowest and highest standards on the linear part of each standard curve on a log-log plot.

For IFNγ the VersaMAP kit had the lowest LLOQ but for IL-17 BioPLEX had the lowest LLOQ. The ULOQ was highest with the BioPLEX kit for both IFNγ and IL-17, however wider linear dynamic ranges were obtained using the MILLIPLEX kit (Table 5.1).

Wells with bead counts <37 were excluded as at least this number is required to minimize the potential impact of outlier beads on median fluorescence intensity (MFI). Low bead counts were more common with the VersaMAP kit (>90% of samples on some runs and up to 1 in 3 standard/control wells). In contrast, low bead counts were not observed in any standard/control wells and in 11% and 1% of samples respectively for the BioPLEX and MILLIPLEX kits. This may have been a result of greater median bead aggregation observed for the VersaMAP kit than for the BioPLEX and MILLIPLEX kits (29% vs 11% and 12% respectively).

Repeatability (precision) was investigated for the BioPLEX and MILLIPLEX kits only.

		CV%			
		Sample 1	Sample 2	Sample 3	Mean
IFNγ	BioPLEX	33.56	33.56	168.17	87.54
	MILLIPLEX	30.45	63.28	35.83	43.19
IL-17	BioPLEX	16.52	6.81	22.66	15.33
	MILLIPLEX	25.65	10.79	23.84	20.09

Table 5.2 Precision of IFNγ and IL-17 measurement by BioPLEX and MILLIPLEX kits. 3 sets of 4 identical spiked supernatants from biopsies disrupted in PBS-based media were included at different positions on the same plate and the % Coefficient of Variation (%CV) calculated for each (mean/SD x 100).

Repeatability was better for IL-17 than for IFNγ. In this analysis the repeatability was better with the MILLIPLEX kit for IFNγ but with the BioPLEX kit for IL-17 (Table 5.2).

Manufacturers have validated Luminex kits for use with serum, plasma and tissue culture supernatants but there are no standard protocols for preparation of tissue samples for Luminex assays. We therefore compared processing methods and extraction buffers using paired biopsies from 4 further patients. Within each pair one biopsy was spiked at 100 pg/ml and the other "unspiked" biopsy with buffer only. The biopsies were then processed, split into aliquots to test using different kits, and frozen at -80°C until analysis.

Manual sample disruption using a mini pellet pestle with or without homogenization using a needle and syringe, and automated processing using a TissueLyser LT bead-basher (QIAGEN) were compared. Cytokine spikes were recovered more accurately from samples processed manually (Table 5.3).

		Median cytokine recovered (pg/ml)				p value for manual vs automated processing
		Manual processing			**Automated processing**	
	Pestle	+	+	+	TissueLyser	
	Needle/syringe		+	+		
	Benzonase			+		
IL-17	BioPLEX	56	51	68	30	0.033
	MILLIPLEX	52	42	62	37	0.12
IFNγ	BioPLEX	143	164	182	9	0.056
	MILLIPLEX	55	41	70	4	0.017

Table 5.3 Comparison of manual and automated processing methods and effect of adding benzonase to the extraction buffer. Gastric mucosal biopsies were spiked with 100pg/ml of IL-17 and IFNγ prior to manual biopsy processing. Data were adjusted for background using paired unspiked biopsies from the same patient, and are equivalent to percentage spiked cytokine recovery.

Additional homogenization using a needle and syringe following disruption with a pestle did not improve cytokine recovery (Table 5.3). Homogenization with a needle and syringe lead to loss of sample volume, which was retained in equipment dead space. Addition of the endonuclease benzonase was also evaluated. Digestion of nucleic acids by benzonase may reduce sample viscosity. There was a trend for increased cytokine recovery when benzonase was included in the extraction buffer but this did not reach statistical significance (Table 5.3).

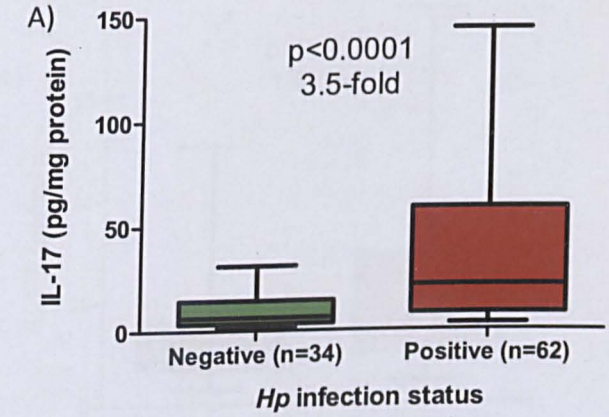
MILLIPLEX kits were used for subsequent analyses as they had demonstrated good accuracy and reasonable sensitivity and precision. They had the advantage of only requiring 25 µl sample, compared to 50 µl for the other kits. MILLIPLEX also have a Th17 panel and other panels available, covering most of the cytokines of interest.

When comparing cytokine concentrations in different gastric biopsies it is necessary to control for biopsy size. This can be done by adjusting for biopsy weight as for the ELISA data in Figure 5.2 and by Serelli-Lee *et al.* (Serelli-Lee et al., 2012). Another approach is to adjust for total protein concentrations measured by either modified Lowry, Bradford or BCA assays (Crabtree et al., 1991, Yamaoka et al., 1997, Yamaoka et al., 2001, Hwang et al., 2002, Shimizu et al., 2004, Yamauchi et al., 2008). Similar to previous studies (Kusugami et al., 1999), the gastric biopsies were small with mean (± SD) weight of 4.3 ± 2.9 mg (n=18). Snap frozen samples are associated with variable amounts of water and mucus during thawing, so weight was a less reliable measure of biopsy tissue content for these samples.

The final processing method selected after optimization comprised: disruption in 300 µL of PBS-based buffer, with benzonase and protease inhibitors, with a pellet pestle on ice, homogenization by repeated aspiration into a 200 µL filter pipette tip to minimize volume loss, incubation on ice, centrifugation and division of the supernatant into aliquots for storage. (further details are given in section 2.3.2.2).

5.3.3 Cytokine Levels in The Gastric Antrum of Patients with *Hp* Infection and Uninfected Controls

Snap frozen gastric biopsies from 62 *Hp*+ and 34 uninfected patients were processed as above and IL-17 concentrations in the supernatants measured by Luminex.



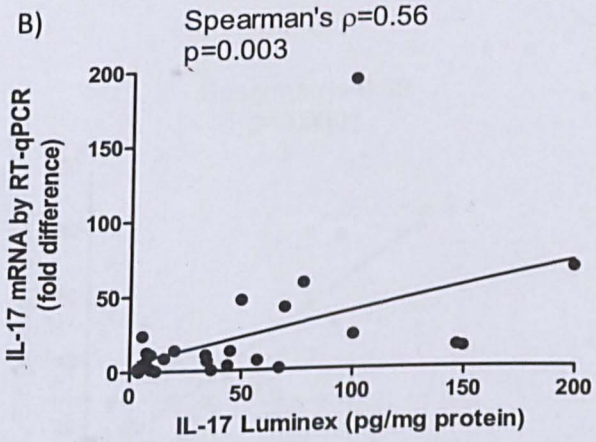


Figure 5.5 IL-17 is increased in the *Hp*-infected stomach and correlates with IL-17 mRNA levels. A) Snap frozen gastric biopsies from 62 *Hp*-infected patients and 34 uninfected controls were processed in PBS-based media and the supernatants assayed for IL-17 by Luminex. B) IL-17 mRNA levels were available for 26 of the *Hp*-infected patients and were correlated with the Luminex results.

In keeping with the RT-qPCR and ELISA results, there was a highly significant increase in IL-17 in the *Hp*-infected stomach (Figure 5.5A). The Luminex and RT-qPCR results correlated positively (Figure 5.5B).

Concentrations of IL-17F, which is also produced by Th17 cells were also measured.

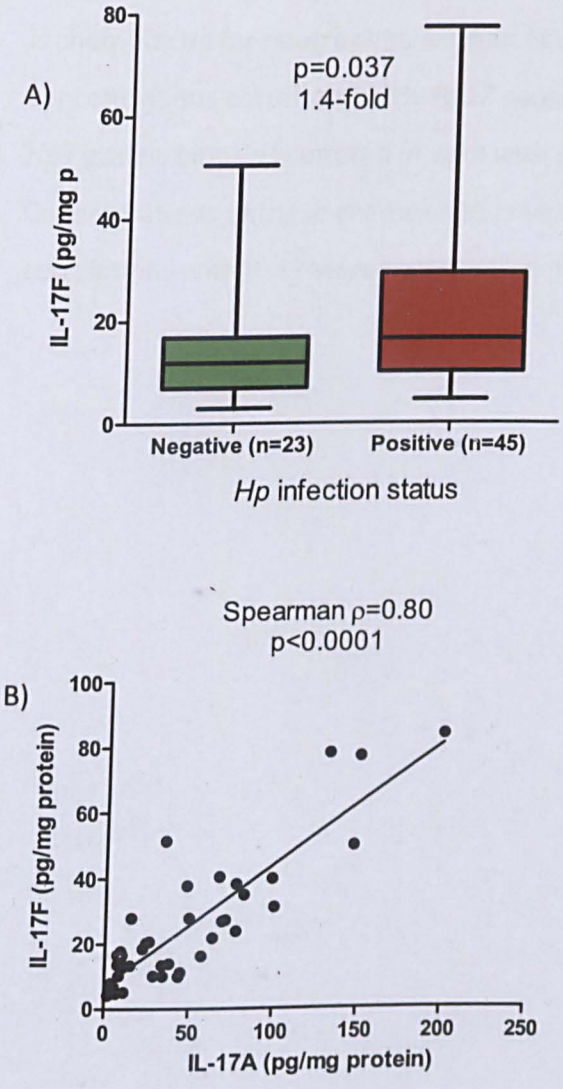


Figure 5.6 IL-17F is increased in the *Hp*-infected stomach and is correlated with IL-17. A) Snap frozen gastric biopsies from 45 *Hp*-infected patients and 23 uninfected controls were processed in PBS-based media and the supernatants assayed for IL-17F by Luminex. B) IL-17 and IL-17F results were correlated for 45 *Hp*+ patients.

IL-17F was significantly increased in the *Hp*-infected stomach and correlated tightly with IL-17 levels (Figure 5.6A and B).

IL-17 can upregulate expression of a number of chemokines including CCL20 and IL-8 by epithelial cells and other cells upon IL-17 receptor binding (Gaffen, 2008). CCL20 is a ligand for CCR6 which is found on Tregs and Th17 cells. IL-8 is chemotactic for neutrophils. Mizuno *et al.* previously reported that IL-8 concentrations correlated with IL-17 concentrations in the supernatants of *Hp*+ gastric biopsies cultured *in vitro* with phytohaemagluttinin for 48 hours. Concentrations of these chemokines in snap frozen gastric biopsies and correlations with IL-17 were investigated next.

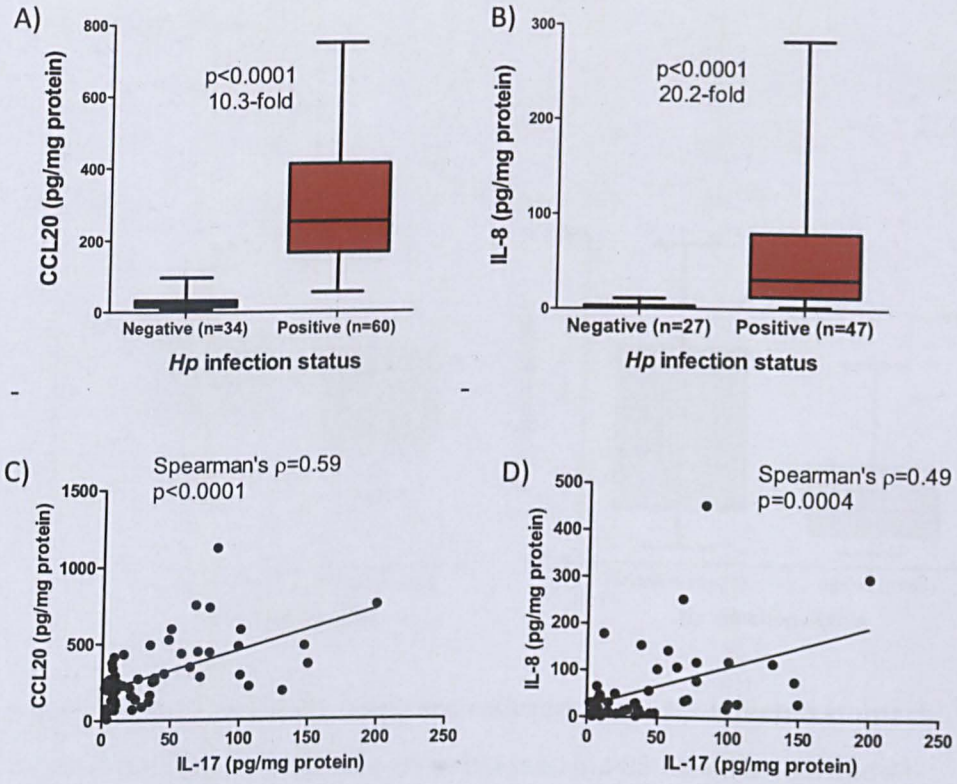


Figure 5.7 The IL-17 target chemokines CCL20 and IL-8 are increased in the *Hp*-infected stomach and correlate with IL-17. Snap frozen gastric biopsies were processed in PBS-based media and A) supernatants from 60 *Hp*-infected patients and 34 uninfected controls assayed for CCL20 and B) supernatants from 47 *Hp*-infected patients and 27 uninfected controls assayed for IL-8 by Luminex. C) IL-17 and CCL20 results were correlated for 58 *Hp*+ patients. D) IL-17 and IL-8 results were correlated for 47 *Hp*+ patients.

There was a highly significant increase in CCL20 and IL-8 in the *Hp*-infected gastric mucosa (Figure 5.7 A and B). CCL20 and IL-8 levels correlated strongly with IL-17 levels (Figure 5.7 C and D).

Levels of IL-22 (often produced by Th17 cells) were not significantly different between *Hp*-infected and uninfected patients (data not shown).

Next concentrations of the Th17-differentiating cytokines IL-21 and IL-23 were quantitated.

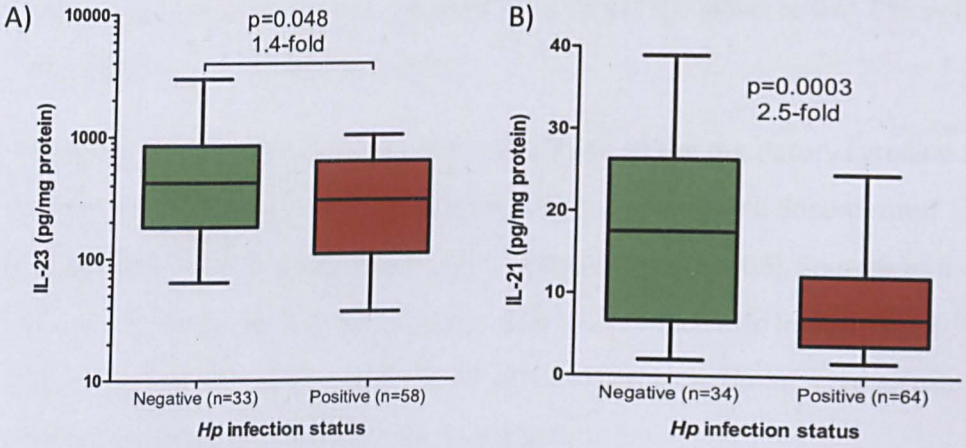


Figure 5.8 IL-23 and IL-21 levels are reduced in the *Hp*-infected stomach. Snap frozen gastric biopsies were processed in PBS-based media and A) supernatants from 58 *Hp*-infected patients and 33 uninfected controls assayed for IL-23 and B) supernatants from 64 *Hp*-infected patients and 34 uninfected controls assayed for IL-8 by Luminex.

There were relatively high levels of IL-23 in all the gastric biopsies. These were unexpectedly slightly higher in the uninfected controls compared to the *Hp*-infected patient biopsies (Figure 5.8A). IL-21 can be produced by Th17 cells and can be involved in their differentiation. IL-21 levels were also significantly higher in the uninfected control biopsies compared to the *Hp*-infected biopsies (Figure 5.8B). There was no significant positive or negative correlation with IL-17 for IL-23 or IL-21 (data not shown).

IL-1β and IL-6 are also involved in human Th17 differentiation but there was no significant difference between either of these cytokines in the *Hp*-infected and uninfected control groups (data not shown).

There was no significant difference in levels of the Th1-related cytokines IL-12p70 and IFNγ between the *Hp*-infected and uninfected groups, though there was a trend towards higher IL-12p70 in the uninfected group (p=0.087). Nor were any significant differences found between the *Hp*-infected and uninfected groups in the pro-inflammatory cytokines TNF-α or GM-CSF or the Th2 cytokine IL-4 (data not shown).

As discussed in section 5.1.3.2., increased levels of the regulatory cytokine IL-10 in the human *Hp*-infected gastric mucosa have been well documented (Bodger et al., 1997, Holck et al., 2003, Robinson et al., 2008). Sources of IL-10 include Tr1 Tregs, which can be induced by IL-27, which also inhibits Th17 differentiation (Pot et al., 2011). IL-27 concentrations in *Hp*-infected gastric mucosa have not previously been investigated.

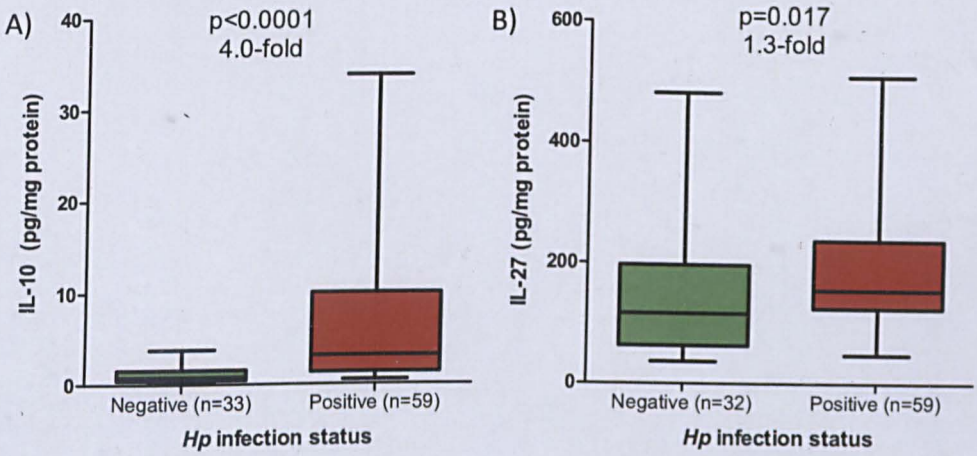


Figure 5.9 IL-10 and IL-27 are increased in the *Hp*-infected stomach. Snap frozen gastric biopsies were processed in PBS-based media and A) supernatants from 59 *Hp*-infected patients and 33 uninfected controls assayed for IL-10 and B) supernatants from 59 *Hp*-infected patients and 32 uninfected controls assayed for IL-27 by Luminex.

IL-10 concentrations were very significantly increased in the *Hp*-infected stomach (Figure 5.9A), consistent with the known increase in Tregs in the *Hp*-infected gastric mucosa. IL-27 was also significantly upregulated in the *Hp*-infected stomach (Figure 5.9B). There was a positive correlation between IL-10 and IL-27 levels (Spearman's rank correlation $\rho=0.51$, $p=0.009$, $n=25$). Tregs express CCR6 and can be recruited by CCL20, however no significant correlation was found between CCL20 and IL-10 concentrations (data not shown).

IL-2 can promote Tregs at the expense of Th17 cells via STAT5 signalling (Laurence et al., 2007) but no significant difference in IL-2 levels between the biopsies from *Hp*-infected and uninfected patients was found (data not shown). TGF-β levels were not measured in this study.

5.3.4 Relative Levels of Th1 and Th17 Cytokines in the *Hp*-Infected Gastric Mucosa

Hp infection in humans is known to cause a Th1 response (section 1.4.2.3) (Robinson et al., 2008, Pellicano et al., 2007) and more recently Th17 responses have been demonstrated in this study (Chapter 6) and by others (Caruso et al., 2008, Serelli-Lee et al., 2012). However, the relative importance of these two types of pro-inflammatory T cell responses is not known. Relative levels of IL-23 and IL-12, which are pro-Th17 and Th1-differentiating factors respectively, and the signature Th17 and Th1 cytokines, IL-17 and IFNγ respectively, in the *Hp*-infected gastric mucosa were therefore compared.

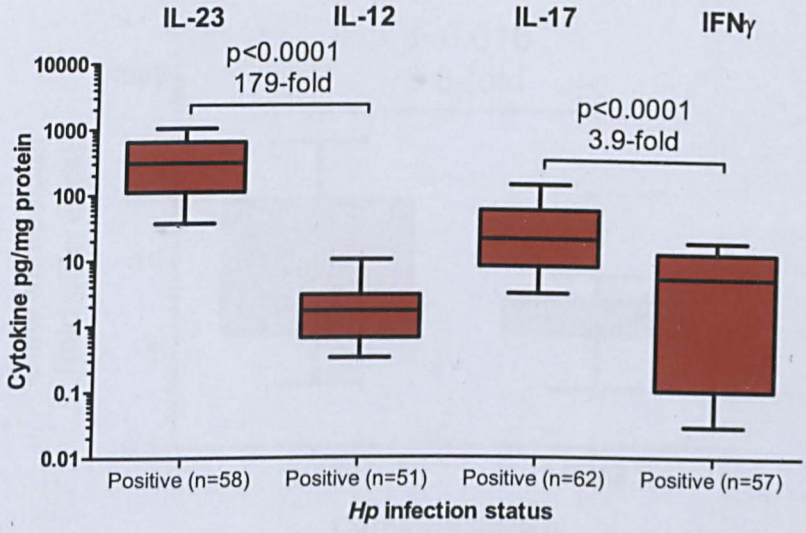


Figure 5.10 Pro-Th17 cytokine IL-23 and Th17 signature cytokine IL-17 are more prevalent than Th1-differentiating cytokine IL-12 and Th1 signature cytokine IFNγ. IL-23 and IL-12p70 levels were measured in supernatants of gastric biopsy supernatants by Luminex as above and relative levels were compared (Mann-Whitney U-test).

IL-23 protein levels were much higher than IL-12p70 protein levels in gastric tissue (Figure 5.10). This was also the case in *Hp*-negative biopsies where there was also 179-fold more IL-23 than IL-12p70 ($p<0.0001$).

In biopsies from *Hp*-infected patients there were 3.9-fold higher levels of IL-17 than the Th1 signature cytokine IFNγ (Figure 5.10). However in biopsies from uninfected patients there was no significant difference in IFNγ and IL-17 levels.

Samples were spiked with known concentrations of IFNγ and IL-12 to confirm that the Luminex assay was able to detect these cytokines satisfactorily.

IL-17 and IFNγ mRNA expression was also compared in 22 *Hp*-infected patients. There was significantly more upregulation of IL-17 mRNA than IFNγ mRNA ($p=0.016$, 3.3-fold) (Figure 5.11).

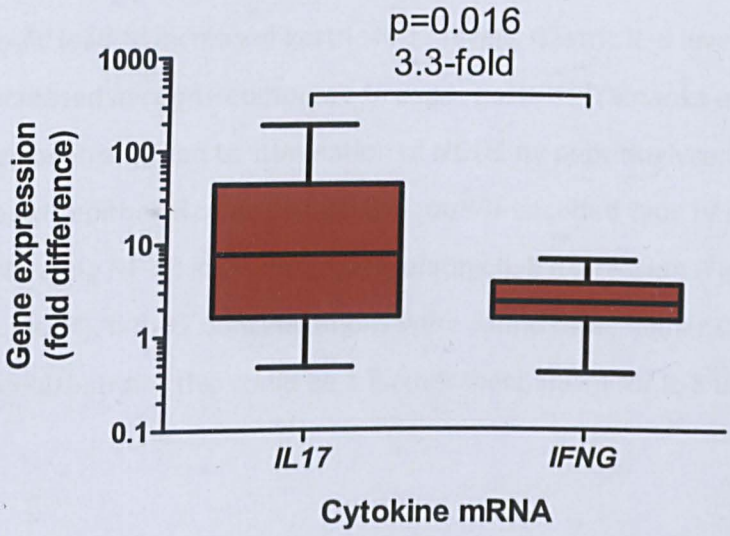


Figure 5.11 IL-17 mRNA expression is higher than IFNγ mRNA expression in the *Hp*-infected gastric mucosa. mRNA was extracted from gastric tissue biopsies from 22 *Hp*-infected patients. Relative expression of the *IL17* and *IFNG* genes was normalized to against GAPDH and expressed relative to a comparator reference sample prepared from 14 uninfected biopsies. Relative expression of the two genes was compared.

The RT-qPCR data was analyzed using the Pfaffl method which takes account of the RT-qPCR efficiency for each gene of interest and the GAPDH used to

normalize (Pfaffl, 2001). RT-qPCR efficiencies for each gene are given in section 2.4.

No correlation was found between IL-17 and IFNγ concentrations at the protein level. This may reflect difficulty accurately measuring low concentrations of IFNγ. There was a strong trend towards a weak positive correlation of *IL17* and *IFNG* mRNA expression, n=25, Spearman's ρ=0.40, p=0.051 (data not shown).

5.3.5 Correlation of IL-17 Levels and *Hp* Virulence Factor Status

Hp strains that are *cagPAI+* are associated with increased risk of both peptic ulcer disease and gastric cancer. MoDCs secreted lower concentrations of IL-12 family cytokines, including IL-23, when stimulated with *Hp* strains with mutated *cagE* (Figure 3.9). This suggests that infection with *cagPAI+* strains could lead to increased gastric IL-17 levels. Gastric IL-8 levels are known to be increased in *cagA+* compared to *cagA-* gastritis (Yamaoka et al., 1999). This has been ascribed to stimulation of NOD1 by peptidoglycan delivered to gastric epithelial cells through the *cagPAI*-encoded type IV secretion system activating NF-κB and hence upregulating IL-8 expression (Figure 1.4) (Viala et al., 2004). If IL-17 concentrations were found to be higher upon infection with *cagPAI+* strains this could be a further mechanism for IL-8 upregulation.

The *cagA* gene was used as a marker for the *cagPAI*. Biopsies from patients infected with *cag*+ strains of *Hp* did not have higher IL-17 levels than those from patients infected with *cag*- *Hp* strains (Figure 5.12A). IL-8 levels were higher in *cag*+ biopsies, as expected (Figure 5.12B).

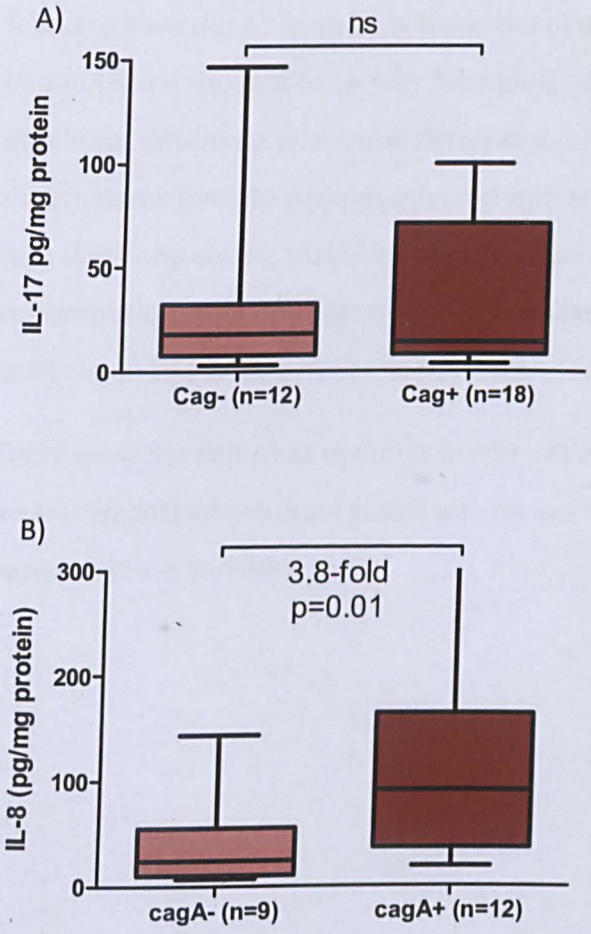


Figure 5.12 IL-17 and IL-8 levels stratified by *Hp* and *cag* status. Biopsies from *Hp* infected patients were homogenized in PBS-based media and A) IL-17 and B) IL-8 levels in the supernatants measured by Luminex. Results were stratified by *cag* status.

The virulence factor *dupA* has been associated with increased risk of duodenal ulceration and higher gastric IL-8 levels *in vivo* (Lu et al., 2005, Hussein et al., 2010). Hussein *et al.* found that monocytes produced less IL-

12p40, IL-12p70 and IL-23 when stimulated by *dupA*-deficient *Hp* strains, suggesting that this virulence factor could work with stimulation of immune cells rather than the gastric epithelium (Hussein et al., 2010). Stimulation of MoDCs with isogenic *dupA* mutants also reduced IL-12p70 and IL-23 secretion (Figure 3.9). IL-17 levels were therefore stratified by *dupA* status to see if this virulence factor is associated with higher gastric IL-17 levels *in vivo*. Patients found to have *dupA2* were included in the *dupA*- group as this truncated form of *dupA* is not thought to be fully functional (Hussein et al., 2010). No significant difference was found between IL-17 concentrations in biopsy supernatants from 10 patients infected with *dupA*- and 5 patients infected with *dupA*+ *Hp* strains (data not shown), however data on both gastric IL-17 concentration and *dupA* status was only available for a small number of patients, so this finding needs to be interpreted with some caution.

There were not sufficient numbers of *Hp*+ patients with IL-8 concentration measurements where *dupA* status was known to assess for an association between these variables.

5.3.6 Association of IL-17 Levels with Histopathological Parameters and Peptic Ulcer Disease

As IL-17 is known to upregulate chemokines which recruit lymphocytes and neutrophils correlations of gastric mucosal IL-17 concentrations with immune cell infiltration were investigated.

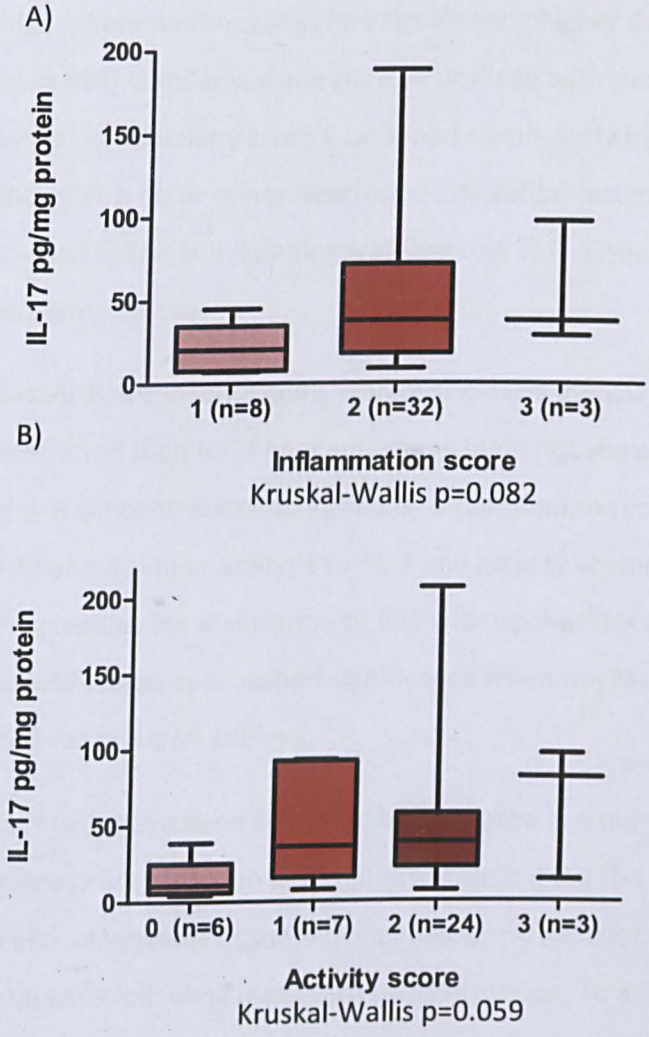


Figure 5.13 Association of IL-17 levels with inflammation and activity scores. Biopsies were taken from 43 *Hp+* patients. One biopsy from each patient was snap frozen, then homogenized in PBS-based media and IL-17 levels in the supernatant measured by Luminex. Further biopsies were scored by an experienced gastrointestinal histopathologist for A) Inflammation score (lymphocyte infiltration) and B) Activity score (neutrophil infiltration). IL-17 levels were stratified by the histopathological parameters.

There were trends for increasing IL-17 with increasing inflammation score (p=0.082) and activity score (p=0.059)(Figure 5.13A and B). As numbers with very high inflammation and activity scores were low (n=3), IL-17 levels in those with low (1) and high (2 or 3) inflammation scores were also compared using the Mann-Whitney U test. Using this method those in the group with high inflammation scores had significantly higher IL-17 concentrations (p=0.024). Similarly, a comparison of those with substantial neutrophil infiltration (activity score 2 or 3) had significantly higher IL-17 levels than those with no or minor neutrophil infiltration (activity score 0 or 1) (p=0.031).This is in keeping with the role of IL-17 in recruitment of neutrophils and lymphocytes.

Gastric IL-8 concentrations were less closely associated with immune cell infiltration than IL-17 concentrations (data not shown). Kruskal-Wallis analysis of IL-8 concentrations stratified by inflammation score resulted in a p value of 0.44 and a similar analysis for IL-8 and activity scores gave a p value of 0.40. The p values for association of IL-8 with neither the inflammation nor activity activity scores approached significance when the Mann-Whitney U-test was used for grouped analysis.

Th17 cells have been shown to be increased in a number of cancers, including gastric cancer (section 1.3.6). It is not clear if the IL-17 response to *Hp* infection contributes to carcinogenesis, or if gastric tumours promote Th17 responses following malignant transformation. To address this question IL-17 levels were stratified by the presence or absence of precancerous changes (atrophy and/or intestinal metaplasia).

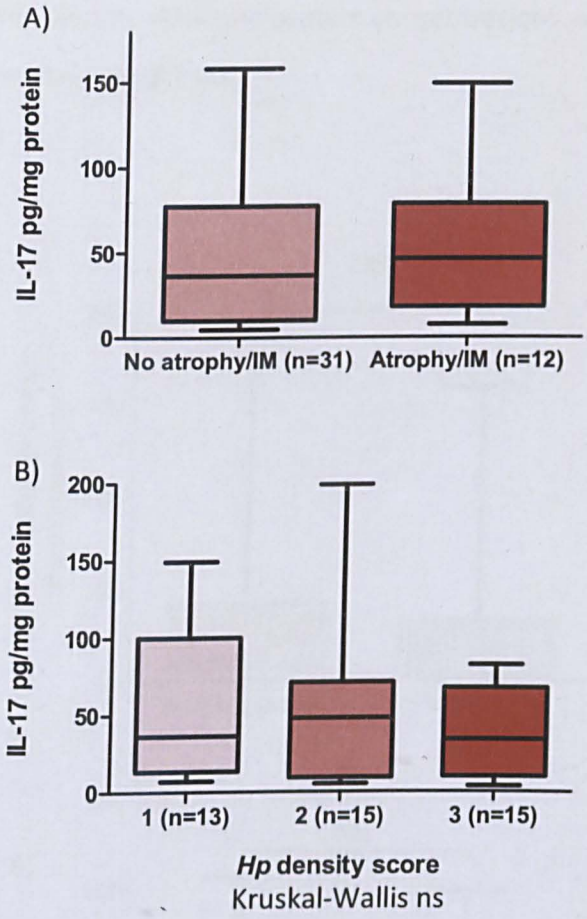


Figure 5.14 IL-17 levels stratified by absence of presence of intestinal metaplasia and/or atrophy and *Hp* density score. Biopsies were taken from 43 *Hp*+ patients. One biopsy from each patient was snap frozen, then homogenized in PBS-based media and IL-17 levels in the supernatant measured by Luminex. Further biopsies were scored by an experienced gastrointestinal histopathologist for A) Atrophy and intestinal metaplasia and B) *Hp* density. IL-17 levels were stratified by the histopathological parameters.

No association was found between IL-17 levels and the presence of precancerous changes (atrophy and/or intestinal metaplasia)(Figure 5.14A), nor was there an association between IL-17 levels and *Hp* density score (Figure 5.14B).

To investigate the hypothesis that Th17-related inflammation contributes to PUD IL-17 mRNA and protein concentrations were stratified by the presence or absence of PUD.

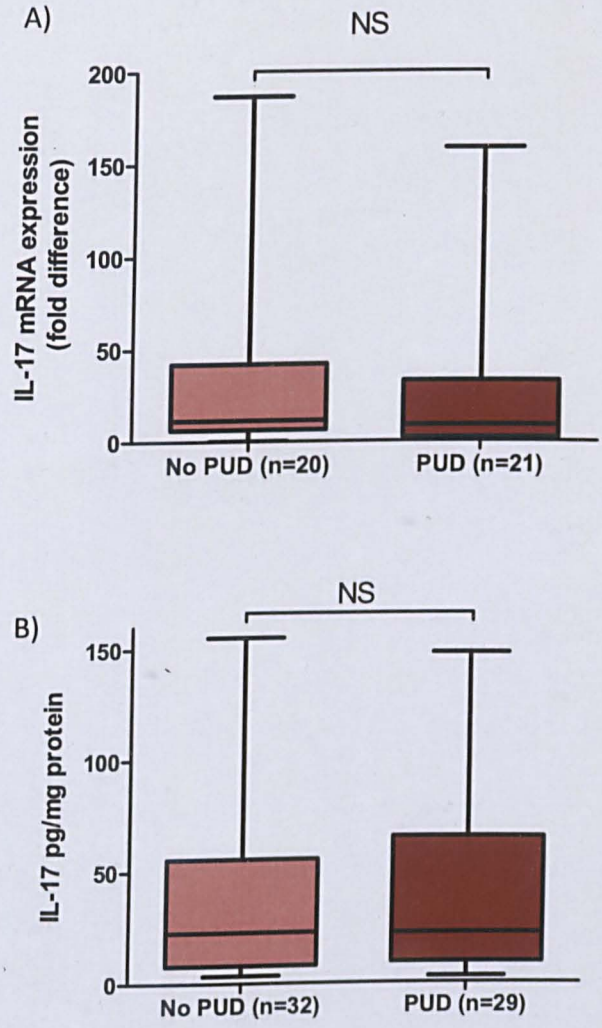


Figure 5.15 IL-17 levels are not increased in peptic ulcer disease. A) mRNA was extracted from gastric tissue biopsies from 41 *Hp*+ patients (20 with and 21 without peptic ulcer disease) and relative *IL-17A* levels calculated by normalizing against *GAPDH* and expressing levels relative to a negative comparator reference sample. B) IL-17 levels were measured in supernatants of gastric biopsy supernatants from 61 *Hp*+ patients (32 with and 29 without peptic ulcer disease) by Luminex as above. IL-17 levels in the presence and absence of peptic ulcer disease were compared.

No significant difference in IL-17 at the mRNA or protein level was found between *Hp*-infected patients with and without peptic ulcer disease (Figure 5.15A and B).

Thankyou to Dr Richard Ingram for RT-qPCR IFNG data and help with Luminex assays.

5.4 DISCUSSION

5.4.1 IL-17 Levels are Increased in the *Hp*-Infected Gastric Mucosa

Increases in IL-17 at the mRNA (Figure 5.2) and protein level (Figure 5.3 and Figure 5.5A) in the *Hp*-infected gastric mucosa have been confirmed using RT-qPCR, ELISA and Luminex techniques. Other studies that have investigated IL-17 mRNA levels in the *Hp*-infected gastric mucosa have not quoted fold differences compared to uninfected controls (Luzza et al., 2000, Caruso et al., 2008, Kimang'a et al.). Here the fold difference in IL-17 mRNA between gastric biopsies from *Hp*-infected patients and uninfected controls (42.6-fold) was much greater than the fold difference in protein levels (6.1-fold and 3.5-fold for ELISA and Luminex respectively). It is possible that not all IL-17 mRNA is translated into protein, or that the protein is degraded more rapidly than the mRNA.

Initially IL-17 protein levels were studied by ELISA, adjusting for the total weight of the biopsies. Serelli-Lee *et al.* measured IL-17 supernatants from a similar number of biopsy culture supernatants using ELISA, adjusted for biopsy weight and also found approximately 6-fold more IL-17 in patients with active *Hp* infection compared to those that had never been infected (Serelli-Lee et al., 2012). Mizuno *et al.* found a similar fold increase in IL-17 concentrations (adjusted for total protein rather than weight) in the supernatants of *Hp*+ gastric biopsies following 48 hours of *in vitro* culture, with higher levels in the gastric ulcer compared to non-ulcer patient biopsies (Mizuno et al., 2005). Subsequently Luminex was used in the current study to maximize data from the gastric biopsies.

5.4.2 Luminex Kit Comparison and Optimization

Gastric biopsies have a variable amount of mucus and water associated with them, so we found that weight did not provide a very reliable measure of the tissue content of the biopsy. Total protein content of biopsies was therefore

measured, using BCA assay and IL-17 results adjusted for total protein levels. Several authors investigating cytokine levels in gastric mucosa have taken the approach of adjusting for total protein levels measured by either modified Lowry, Bradford or BCA assays (Crabtree et al., 1991, Yamaoka et al., 1997, Yamaoka et al., 2001, Yamauchi et al., 2008).

Analyzing cytokine levels in the supernatants of snap frozen homogenized gastric biopsies should reflect the inflammatory state of the gastric mucosa at the time of biopsy. This would be expected to more directly reflect the actual cytokine levels present than studying their mRNA levels or measuring cytokines after *in vitro* culture. Many cytokines are only present at low concentration in the gastric mucosa, so measuring their levels in small mucosal biopsies presents a technical challenge. ELISA usually requires at least 100 μl sample for each cytokine to be measured. Often it was necessary to use more than one gastric biopsy to obtain IL-17 levels within the range of the assay, especially for uninfected patients.

Luminex allows multiplexing so that many cytokines can be measured in 25-50 μl sample in a single assay. In addition to allowing data on many more cytokines to be obtained from valuable gastric biopsies this allows direct comparison of levels of different cytokines in the same sample. Sensitivity is similar to ELISA but biopsies can be homogenized in a small volume of extraction buffer, thereby increasing cytokine concentrations.

All Luminex methods have in built replicates in the sense that the quantification reaction occurs on the surface of multiple beads for each analyte in each well, which are analyzed independently.

RPMI-based extraction buffer gave some very high background IFNγ readings with the BioPLEX kit. PBS-based extraction buffer was therefore used for the main study.

The BioPLEX kit was the fastest assay to run with the longest incubation time only 30 minutes. Both the VersaMAP and MILLIPLEX kits had incubations of 2

hours after adding the samples, then 1 hour after adding the biotinylated antibody.

The MILLIPLEX kit had the advantage of only requiring 25 ul sample, whereas the VersaMAP and BioPLEX kits required 50 ul sample. It was also relatively accurate and reasonably sensitive and precise. Most of the analytes of interest were available for MILLIPLEX so this was used for further analysis.

5.4.3 Cytokine Levels in Gastric Antral Biopsies from Patients with *Hp* Infection and Uninfected Controls

IL-17 levels were measured in 62 *Hp*-infected patients and 34 uninfected patients and were found to be 3.5-fold higher in those with *Hp* infection. This is the largest study of IL-17 protein levels in *Hp* infection to date. IL-17 levels were not measured by Luminex in the biopsy supernatants used for ELISA, so a direct comparison of IL-17 ELISA and Luminex results was not possible.

IL-17F protein was shown to be increased in *Hp*-infected gastric biopsies for the first time. IL-17F correlated strongly with IL-17 levels. This correlation has been shown previously at the mRNA level (Kimang'a et al.), and might be expected as IL-17 and IL-17F are both produced by Th17 cells.

The chemokine CCL20 is important for attracting $CCR6^+$ Th17 cells and Tregs. Its expression by epithelial cells is upregulated by IL-17, IL-β, TNFα and IL-21 (Kao et al., 2005, Caruso et al., 2007b, Wu et al., 2007). Murine peritoneal macrophages cultured with *Hp* also upregulated CCL20 (Zhuang et al.). T cells in the gastric mucosa of *Hp*-infected patients express high levels of CCR6 compared to peripheral blood T cells from the same patients (Duerr et al., 2006), suggesting that $CCR6^+$ cells are preferentially recruited to the inflamed stomach. Others have previously demonstrated increased CCL20 in the *Hp*-infected human gastric mucosa (Yoshida et al., 2009, Serelli-Lee et al., 2012, Duerr et al., 2006) but this is the first time that it has been shown to correlate with IL-17 levels. CCL20 has anti-microbial effects against organisms including *Escherichia coli, Pseudomonas aeruginosa, Moraxella catarrhalis,*

Streptococcus pyogenes, Enterococcus faecium, Staphylococcus aureus and *Candida albicans* (Yang et al., 2003). However it is associated with chronic gastritis in *Hp* infection (Yoshida et al., 2009).

IL-8 is thought to have a key role in *Hp*-induced pathology due to its neutrophil-attracting properties and is probably the most extensively studied cytokine in *Hp* research. It was therefore helpful to include it in this study to validate the methods used against previously published data as well as to confirm the association between IL-8 and IL-17. Numerous other studies have found highly significant increases in IL-8 protein during *Hp* infection, but most have used semi-quantitative immunohistochemistry (Holck et al., 2003) or ELISA which found median IL-8 concentrations in uninfected controls to be 0 pg/ml (Yamaoka et al., 1997, Yamaoka et al., 1998), making it impossible to calculate a fold difference, or values were not given (Shimizu et al., 2004). A recent study by Serelli-Lee *et al.* used Luminex and found that IL-8 was 46-fold increased in biopsies from *Hp*-infected gastric biopsies compared to those from uninfected controls. This study found a smaller, 20-fold difference, but Serelli-Lee *et al.* adjusted for biopsy size using weight whereas total protein levels were used here so the results are not directly comparable.

Mizuno *et al.* previously reported a strong correlation between IL-8 and IL-17 levels in supernatants of gastric biopsies cultured with phytohaemagluttinin for 48 hours. Strong correlation between these cytokines was evident again here in snap frozen gastric biopsies.

Given the marked increase in IL-17 concentrations in the *Hp*-infected gastric mucosa levels of other Th17 effector cytokines were also investigated. No significant difference was found in IL-22 levels between *Hp*-infected biopsies and those from uninfected controls. This is consistent with recent immunohistochemistry and ELISA findings by Serelli-Lee *et al.* (Serelli-Lee et al., 2012). Th17 cells are not all the same. The cytokines they produce may vary depending on the stimulating pathogen and local factors such as availability of AhR ligands (Zielinski et al., 2012, Veldhoen et al., 2008a,

Quintana et al., 2008, Veldhoen et al., 2009). Mouse studies indicate that IL-22 present in mucosal tissue may have roles in homeostasis and tissue repair (Zenewicz et al., 2008, Sugimoto et al., 2008, Pickert et al., 2009). It is possible that even in the absence of an additional *Hp*-induced IL-22 response, this baseline IL-22 can synergize with IL-17 to promote anti-bacterial immunity (Liang et al., 2006).

High levels of IL-23 were found in the gastric mucosa, as previously reported (Caruso et al., 2008). However, in contrast to the findings of Caruso *et al.*, no increase in IL-23 was found in the gastric mucosa of *Hp*-infected patients in this study, in fact their IL-23 levels were slightly lower than those found in uninfected controls. Increased IL-23p19 mRNA has also been reported in the *Hp*-infected gastric mucosa (Caruso et al., 2008, Serelli-Lee et al., 2012). No mention of exclusion of patients taking PPIs or antibiotics was made in the study by Caruso *et al.*, which could have led to failure of detection of *Hp* infection in some of the patients classified as *Hp* negative. As gastric IL-23 levels are so high in both infected and uninfected patients it is unlikely that this cytokine will be a limiting factor restraining T cell differentiation.

Caruso *et al.* previously reported increased IL-21 in *Hp*-infected gastric biopsies by RT-qPCR and western blotting. This might be expected, as IL-21 is often produced by Th17 cells and can promote their differentiation, but contrasts with the findings of this study which detected a highly significant reduction in IL-21 protein levels in gastric biopsies of *Hp*-infected patients compared to uninfected controls. As in the other study by Caruso *et al.* discussed above, patients taking proton pump inhibitors or antibiotics were not excluded, which could have led to some misclassification of *Hp* status. Raw IL-21 data from *Hp*+ and uninfected biopsies were compared to exclude the possibility that high total protein levels in the *Hp*+ biopsies were causing spuriously low adjusted IL-21 levels. Raw IL-21 levels were also significantly lower in the *Hp*+ biopsies (there was a non-statististically significant trend towards higher total protein levels in the *Hp*+ biopsies). IL-21 can also be produced by the Tr1 group of regulatory T cells (Apetoh et al., 2010, Pot et al.,

2011) and may have an anti-inflammatory role. However, IL-10 secreting Tregs are increased in the *Hp*-infected human stomach (Robinson et al., 2008), so this does not explain the reduced IL-21 levels found in the *Hp*-infected gastric biopsies here.

In keeping with published immunohistochemistry, flow cytometry and PCR data (Lindholm et al., 1998, Holck et al., 2003, Robinson et al., 2008), there was a highly significant increase in IL-10 levels in the *Hp*-infected compared to uninfected biopsies. Much of this IL-10 is likely to be derived from $CD4^{+}CD25^{high}$ Tregs (Amberbir et al., 2011, Robinson et al., 2008). IL-27 was also shown to be increased in the *Hp*-infected stomach for the first time. This is consistent with its role in inducing IL-10 secreting regulatory T cells (Fitzgerald et al., 2007, Stumhofer et al., 2007, Awasthi et al., 2007, Pot et al., 2011). IL-2 has the potential to shift the balance between Th17 cell and Tregs but there was no significant difference in IL-2 between the *Hp*-infected and uninfected groups.

5.4.4 Relative Levels of Th1 and Th17 Cytokines in the *Hp*-Infected Gastric Mucosa

Though the presence of Th1 and Th17 cytokine responses in the human *Hp*-infected gastric mucosa have been reported previously (Lindholm et al., 1998, Pellicano et al., 2007, Luzza et al., 2000) little has been published about their relative magnitudes. IL-17 levels were 3.9-fold higher than IFNγ levels in gastric biopsies from *Hp*-infected patients (unpaired analysis), suggesting that the Th17 response predominates. Samples were spiked with known concentrations of IFNγ and IL-12 to confirm that the Luminex assay was able to detect these cytokines satisfactorily. The gastric IFNγ concentrations detected were of a similar order of magnitude to those found in a study of *Hp* infected children by Shimizu *et al.* (Shimizu et al., 2004).

Greater upregulation of IL-17, compared to IFNγ, was also confirmed at the mRNA level by RT-qPCR. Pro-Th17 cytokine IL-23 was 179-fold more prevalent than the Th1-differentiating cytokine IL-12 in the *Hp*+ gastric biopsies. This

again suggests a Th17 bias, although other cytokines are also involved in Th17 and Th1 differentiation (Manel et al., 2008, Wong et al., 2009, Acosta-Rodriguez et al., 2007a, Volpe et al., 2008). IL-23 levels were also significantly higher than IL-12 levels in the uninfected gastric mucosa.

In a recent study Serelli-Lee *et al.* used ELISAs to measure IL-17 and IFNγ. Lamina propria mononuclear cells were cultured with phytohaemagglutinin and IL-2 and culture supernatants assayed. Biopsy weight rather than total protein was used to adjust for biopsy size, so the results are not directly comparable. In contrast to findings of the current study Serelli-Lee *et al.* reported median IL-17 concentrations 89 pg/ml/g tissue and IFNγ concentrations 173 pg/ml/g tissue. The *in vitro* culture or other methodological differences with the current study could account for the discrepant findings. It is also possible that differences in the study populations could have contributed to the differences.

Relative levels of IL-12(p70) and IL-23 produced by DCs co-cultured with *Hp in vitro* were investigated in Chapter 3. MoDCs produced much more IL-12(p70) than IL-23, and naïve $CD4^+$ T cells co-cultured with the *Hp*-primed MoDCs had corresponding high IFNγ responses. MyDCs co-cultured with *Hp* produced low levels of IL-23 but undetectable IL-12(p70), yet naïve $CD4^+$ T cells co-cultured with the *Hp*-primed MyDCs produced more IFNγ than IL-17 (Chapter 3). This strong *in vitro* IFNγ response is at variance with the Luminex findings from snap frozen gastric biopsies and RT-qPCR findings. This raises questions as to how well the *in vitro* DC models reflect the gastric mucosa *in vivo*. In contrast to the *in vitro* situation where only DCs and naïve $CD4^+$ T cells were present, supernatants of snap frozen gastric biopsy homogenates will contain cytokines derived from additional cell types, including epithelial cells and innate immune cells. IL-17 and IFNγ can be produced by $CD8^+$ T cells, NK cells, NKT cells, γδ T cells and other innate-like cells such as ILCs including LTis, which could be present in the gastric mucosa, in addition to conventional $CD4^+$ T cells. Simple *in vitro* models cannot entirely mimic the complexity of

the gastric mucosa where multiple cell types interact and tissue factors may also influence $CD4^+$ T cell differentiation. The source of IL-17 in the *Hp*-infected gastric mucosa is investigated in Chapter 6.

5.4.5 Correlation of IL-17 Levels and *Hp* Virulence Factor Status

Neither *cagA* nor *dupA* status correlated with gastric IL-17 levels, though numbers studied were low, particularly for *dupA* status so an association cannot be completely excluded. IL-8 concentrations were higher in biopsies infected with cagA+ *Hp* strains, as expected, validating the methods used. The lack of IL-17 correlation with virulence factor status is in keeping with the lack of direct association found between IL-17 and peptic ulcer disease or precancerous changes. It is possible that localized, rather than total biopsy, IL-17 concentrations are important for the development of disease, or that IL-17 has a role at a particular stage of disease (see below). It is now recognized that Th17 cells are heterogeneous. Some may co-secrete IL-10 and have a regulatory phenotype, whilst others may co-express IFNγ and/or GM-CSF and be more pro-inflammatory (Peters et al., 2011). This may cloud the picture regarding the role of IL-17.

5.4.6 Association of IL-17 Levels with Histopathological Parameters and Peptic Ulcer Disease

5.4.6.1 Association of IL-17 concentrations with inflammation and activity scores

As IL-17 protein levels were measured in gastric biopsies from a large number of *Hp*+ patients in this study it was possible to assess for associations between gastric IL-17 levels and histopathological scores and clinical features. High inflammation scores (measure of mononuclear cell infiltration) and high activity scores (measure of neutrophil infiltration) were associated with higher IL-17 levels upon grouped analysis, though the trends did not quite reach significance when analyzed using Kruskal-Wallis (Figure 5.13A and B).

Given the role of Th17 cells in the recruitment of these types of immune cell to sites of inflammation this association might be expected. IL-8 concentrations did not correlate with inflammation or activity scores. Mizuno *et al.* previously showed that IL-17 levels in gastric biopsy culture supernatants correlated more strongly with mononuclear cell infiltration and neutrophil infiltration than IL-8 levels (Mizuno et al., 2005). In this study neutrophils and monocytes were quantified, whereas here the category-based Sydney scoring system was used.

5.4.6.2 Association of IL-17 concentrations with precancerous changes

Zhang *et al.* found increased Th17 cells in the peripheral blood of gastric cancer patients, with higher levels in those with advanced stage cancer. They also detected increased Th17 cells in the tumour draining lymph nodes and increased serum IL-17 in patients with advanced gastric cancer (Zhang et al., 2008a). Iida *et al.* found higher IL-17 mRNA levels in gastric cancer tissue compared to normal adjacent tissue, which were higher in tumours of patients with more advanced stage disease (Iida et al., 2011). This study also reported that most $CD4^+$ cells in gastric tumours co-stained for IL-17 using immunohistochemistry (Iida et al., 2011). Increased IL-17 has also been reported in a number of other types of malignancy including ovarian, breast, hepatocellular, renal, pancreatic and prostate cancers (Kato et al., 2001, Zhu et al., 2008, Zhang et al., 2009, Kryczek et al., 2007, Steiner et al., 2003).

The findings of increasing IL-17 and Th17 cells in tumour draining lymph nodes in gastric cancer suggest that Th17 cells/IL-17 might have a role in carcinogenesis, but it is also possible that the tumour microenvironment promotes IL-17 production following neoplastic transformation. Su *et al.* studied the effect of tumour cell lines and tumour-derived fibroblast cell lines on naïve $CD4^+$ T cells to help address this issue. Both cell lines increased the number of Th17 cells when naïve $CD4^+$ T cells from peripheral blood were used, though the increase was more marked with the tumour-derived

fibroblasts (Su et al., 2010). However, the same cell lines did not increase Th17 cells when naïve $CD4^+$ T cells from cord blood were used, suggesting the Th17 cells in cancer may be from recruitment and expansion of existing Th17 cells rather than *de novo* differentiation of completely naïve T cells. In the same study attraction of Th17 cells by supernatants from tumour cell and tumour-derived fibroblast cell lines was reduced by antibodies against MCP-1 and RANTES. Expression of these chemokines by tumour cell lines was increased upon stimulation with TLR ligands and the NOD2 ligand muramyl dipeptide (Su et al., 2010).

The literature on the role of IL-17 in cancer is mixed. IL-$17^{-/-}$ mice have been reported to have more rapid tumour growth (Kryczek et al., 2009b, Martin-Orozco et al., 2009), but other studies have reported slower tumour growth in IL-$17^{-/-}$ and IL-$17R^{-/-}$ mice (Wang et al., 2009, He et al., 2010). IL-17 can promote angiogenesis (Numasaki et al., 2003) and studies have found that high levels of IL-17-producing cells in hepatocellular carcinoma and non-small cell lung patients (measured by immunohistochemistry) are associated with increased tumour microvessels and reduced survival (Zhang et al., 2009, Chen et al., 2009). On the other hand ovarian cancer patients with higher ascitic fluid IL-17 levels had increased survival (Kryczek et al., 2009b). Mouse melanoma IFNγ-secreting Th17 cells and $CD8^+$ IL-17-secreting "Tc17" cells can mediate anti-tumour immunity (Garcia-Hernandez et al., 2010, Muranski et al., 2008, Hinrichs et al., 2009). The Th17 and Treg lineages are closely related. As Tregs can downregulate anti-tumour immunity, the balance between Tregs and Th17 cells in the tumour microenvironment may be critical. Gnerlich *et al.* demonstrated that adding IL-6 to murine pancreatic cancer skewed the balance, increasing Th17 cells numbers, reducing tumour growth and increasing survival (Gnerlich et al., 2010).

IL-17 may be produced by cells other than Th17 cells in the tumour microenvironment, including $CD8^+$ T cells and macrophages (Kryczek et al., 2007, Zhu et al., 2008). Th17 cells in tumours produce other cytokines in

addition to IL-17. The phenotype and function of tumour infiltrating Th17 cells is discussed further in section 1.3.6.

Studying gastric biopsies from patients with precancerous changes may help to elucidate whether IL-17 has a role in carcinogenesis. As patients with the precancerous changes of atrophy and intestinal metaplasia were uncommon in our cohort, patients with either or both of these histopathological changes were combined. No significant difference was found between gastric mucosal IL-17 levels in those with and without precancerous changes (Figure 5.14A). Similarly, Mizuno *et al.* did not find any association between IL-17 levels secreted in their gastric biopsy culture system and degree of atrophy or intestinal metaplasia (Mizuno et al., 2005). Though numbers are relatively small, this suggests that IL-17 may not have a significant role in initiation of gastric carcinogenesis but may modulate tumour growth once neoplastic transformation has occurred.

5.4.6.3 IL-17 concentrations and Hp *density scores*

No association was found between IL-17 levels and *Hp* density (Figure 5.14B). An earlier study by Luzza *et al.* positively correlated IL-17 mRNA, as well as IFNγ, IL-12 and IL-8 mRNA levels with *Hp* density in children (Luzza et al., 2001). This study did not find any correlation between inflammation or activity scores and IL-17 levels in the *Hp*-infected children but numbers were quite low (n=13). It is clear that the human host mounts an IL-17 response to *Hp* infection but IL-17 promotes a range of anti-bacterial responses, including anti-bacterial peptide expression, recruitment of lymphocytes, neutrophils and other phagocytes (Liang et al., 2006, Gaffen, 2008). Presumably in chronic *Hp* infection in the adult human stomach a balance is reached between the bacteria provoking an IL-17 response and the IL-17 limiting bacterial numbers.

5.4.6.4 Association of IL-17 concentration and peptic ulcer disease

Neither IL-17 mRNA levels nor IL-17 protein were associated with peptic ulcer disease (Figure 4.15A and B). Nor was an association found between GM-CSF

or TNF-α, which can be produced by Th17 cells, and occurrence of peptic disease in this study. Previous data on IL-17 and peptic ulcer disease is limited. Mizuno *et al.* measured IL-17 levels in gastric biopsy culture supernatants and also found no significant difference in levels between their *Hp*-infected peptic ulcer and ulcer-free groups (Mizuno et al., 2005). However, in the same study IL-17 levels in the supernatants of biopsies taken from the gastric ulcer site were higher than those in the supernatants of biopsies taken from the antrum of the same patients, suggesting that IL-17 may contribute locally to mucosal inflammation and breakdown. Jafarzadeh *et al.* found higher serum IL-17 levels in *Hp*-infected patients with duodenal ulcers compared those with asymptomatic infection (Jafarzadeh et al., 2009).

The relationship between Th17 cells and peptic ulcer disease and the cellular sources of gastric IL-17 will be investigated in Chapter 6.

CHAPTER 6

TH17 AND OTHER IL-17-PRODUCING CELLS IN THE GASTRIC MUCOSA

6. TH17 AND OTHER IL-17-PRODUCING CELLS IN THE GASTRIC MUCOSA

6.1 INTRODUCTION

6.1.1 Gastric Th17 Response to *Hp*

As shown previously by others (Luzza et al., 2000, Caruso et al., 2008, Serelli-Lee et al., 2012) and confirmed in Chapter 5 (Figures 5.2, 5.3 and 5.5), IL-17 is significantly increased in the human *Hp*-infected gastric mucosa. However, there is little published data on which cells produce the IL-17. Caruso *et al.* found increased numbers of both $CD4^+$ and $CD8^+$ IL-17-producing T cells in the *Hp*-infected mucosa by flow cytometry, though this was a small study with only 5 *Hp*+ subjects and $CD8^+$ T cells were not positively identified (Caruso et al., 2008). Serelli-Lee *et al.* found that $CD4^+IL\text{-}17^+$ cells identified by immunofluorescence microscopy were increased in those with previous *Hp* infection compared to those who had never been infected, but the difference between those with active infection and those who had never been infected was not significant (Serelli-Lee et al., 2012).

Th17 responses to *Hp* have been better studied in mice. *Hp* urease subunit B promoted Th17 responses *in vitro* and when used to immunize mice (Zhang et al., 2011). There is evidence that Th17 cells are protective in mouse vaccination models (Velin et al., 2009, DeLyria et al., 2009), though it has also been reported that vaccinated mice can be immune to *Hp* challenge in the absence of IL-17 (DeLyria et al., 2011). IL-17/Th17 responses precede IFNγ/Th1 responses in mice (Algood et al., 2007, Shi et al., 2010). $IL\text{-}23^{-/-}$ mice had increased levels of *Hp* but reduced IL-17, IFNγ and inflammation (Horvath et al., 2012). Th1 responses were also reduced in $IL\text{-}17^{-/-}$ mice, suggesting that Th17 and Th1 responses to *Hp* may act synergistically (Shi et al., 2010).

Th17 cells are the best characterized producers of IL-17, but as discussed in Section 1.3.9, a number of other cell types can act as sources of IL-17. These include other types of T cells, such as $CD8^+$ T cells, γδ T cells and NKT cells and

non-T cell innate lymphoid sources including LTi-like cells and other members of the recently designated ILC3 group (Billerbeck et al., 2010, Huber et al., 2009, Kondo et al., 2009, Ortega et al., 2009, Sutton et al., 2009, Martin et al., 2009, Rachitskaya et al., 2008, Doisne et al., 2009, Takatori et al., 2009, Buonocore et al., 2010).

Here cells from *Hp*+ and uninfected gastric biopsies were stained with CD3, CD4, CD8 and IL-17 and analyzed by flow cytometry, allowing identification of Th17, Tc17 and $CD4^{-}CD8^{-}IL\text{-}17^{+}$ T cells.

6.1.2 The Role of Th17 and Other $CD4^{+}$ T cell Subsets in *Hp*-induced Disease

The few studies of the human Th17 response to *Hp* have not investigated associations with disease, although Th17 responses are known to contribute to pathology in *Hp*-infected mice (Shi et al., 2010, Horvath et al., 2012). In this chapter the Th17 marker *RORC2* is correlated with histopathological findings and disease giving novel insights into the role of Th17 cells in *Hp*-induced disease in humans.

$CD4^{+}$ T cells are required for development of gastric pathology in mouse *Hp* and *H. felis* infection models (Eaton et al., 2001, Roth et al., 1999). Nagai *et al.* found that transfer of naïve $CD4^{+}$ T cells into $Rag2^{-/-}$ mice two months after *Hp* infection induced gastritis (Nagai et al., 2007). A recent study by Hitzler *et al.* confirmed that α/β T cells are required to reduce *H. felis* colonization and for development of *H. felis*-induced precancerous changes (Hitzler et al., 2012a).

Th1 responses to *Hp* have been associated with pathology, as described below, but the relative importance of Th1 and Th17 responses for the development of disease is not clear. Current knowledge of the roles of these populations in peptic ulcer disease and gastric cancer is reviewed below.

6.1.2.1 $CD4^{+}$ T cell response to Hp and peptic ulcer disease

As discussed in Chapter 5, IFNγ responses to *Hp* are associated with gastritis in the infected human gastric mucosa and gastritis and peptic ulcer disease in

Hp-infected mice and Mongolian gerbils (Sawai et al., 1999, Lindholm et al., 1998, Lehmann et al., 2002, Holck et al., 2003, Pellicano et al., 2007, Smythies et al., 2000, Akhiani et al., 2002, Yamaoka et al., 2005). D'Elios *et al.* found that T cell clones derived from gastric tissue of *Hp*+ patients with peptic ulcer disease had a predominantly Th1 response to *Hp* antigen stimulation, whereas in those from *Hp*+ patients with gastritis only a Th0 (IL-4 and/or IL-5 with IFNγ) response predominated (IL-17 responses were not assessed) (D'Elios et al., 2003). Higher numbers of $CD4^{+}FOXP3^{+}$ T cells were found in areas of gastric metaplasia in the duodenum (Kindlund et al., 2009), which could contribute to persistence of *Hp* at high colonizations and risk of ulceration. Robinson *et al.* found that patients with peptic ulcers had increased levels of Th1 (and Th2) cells in their gastric mucosa compared to those without ulcers (Robinson et al., 2008). Again Th17 cells were not investigated in this study. As might be expected, Tregs showed the inverse, with strong Treg responses being associated with reduced risk of peptic ulcer disease (Robinson et al., 2008, Hussain, 2012).

6.1.2.2 $CD4^{+}$ T cell response to Hp and neoplastic disease

Hp was listed as a grade I carcinogen by the World Health Organization in 1994 and is a major cause of gastric adenocarcinoma and gastric lymphoma (IARC, 1994). Recent studies using TCR-αβ knock-out mice indicate that classical T cells are required for the development of preneoplasia, however preneoplastic lesions could develop in $p35^{-/-}$ and $p19^{-/-}$ mice (Hitzler et al., 2012a). T-$bet^{-/-}$ mice were protected against the development of cancer in the *H. felis* murine infection model, suggesting a key role for Th1 cells in gastric carcinogenesis (Stoicov et al., 2009). Interestingly the T-$bet^{-/-}$ mice still had substantial levels of IFNγ, albeit lower than the wild-type controls in this study and other cytokines including IL-1β, TNF-α and IL-10 were also reduced in the T-$bet^{-/-}$ mice (Stoicov et al., 2009). IFNγ expressed by Th1 cells appears to have the dual role of reducing *Hp* burden and promoting carcinogenesis (Sayi et al., 2009, Mueller et al., 2009).

Th17 cells are increased in the peripheral blood of patients with gastric cancer, and levels increase with clinical stage of disease (Zhang et al., 2008a). However, their role in initial transformation to *Hp*-induced gastric cancer is not clear. Iida *et al.* found increased IL-17 mRNA in tumour tissue compared to adjacent normal tissue, and high IL-17 levels were associated with markers of progression, but all the patients in this study had established gastric cancer (Iida et al., 2011). Hitzler *et al.* used two different mouse infection models and found that precancerous changes were reduced in $p19^{-/-}$ mice infected with *Hp* (PMSS1 strain) but there was no difference in precancerous changes between the two groups of mice following *H. felis* infection (Hitzler et al., 2012a).

It is well established that IL-1β polymorphisms are associated with increased risk of gastric cancer (El-Omar et al., 2000). IL-1β promotes Th17 differentiation, so some of the pro-carcinogenic effects of IL-1β could be mediated via Th17 cells. Serelli-Lee *et al.* found that Th17 cells were elevated in patients who had previously had *Hp* infection and IL-17 and IL-1β levels remained elevated despite *Hp* eradication (Serelli-Lee et al., 2012). When IL-1β was neutralized the $CD4^+$ T cell IL-17 response to *Hp* antigen stimulation was reduced, leading the authors to suggest that IL-1β may drive a persistent Th17 response, which could account for the ongoing risk of gastric cancer following *Hp* eradication (Serelli-Lee et al., 2012). $IL\text{-}1R^{-/-}$ mice were protected against *Hp*-induced preneoplastic changes (Hitzler et al., 2012b). Polymorphisms in *IL17* itself and the Th1-related genes *TNFA* and *IFNGR2* have also been associated with increased gastric cancer risk (Hou et al., 2007, Shibata et al., 2009).

Increased numbers of $CD4^+FOXP3^+$ were found in gastric cancer compared to adjacent normal mucosa (Enarsson et al., 2006, Jang, 2010). These Tregs could be suppressing anti-*Hp* and anti-tumour immunity, but studies in gastric cancer patients cannot elucidate whether Tregs are involved in carcinogenesis or favoured by the tumour microenvironment. Tregs can suppress anti-tumour immunity. High Treg frequencies and high FOXP3:CD8 ratios occur in

advanced stage gastric cancer and are associated with poor prognosis (Shen et al., 2010, Mizukami et al., 2008). The distribution of the Tregs can also affect prognosis (Mizukami et al., 2008). My colleagues found increased $CD4^{+}CD25^{high}IL\text{-}10^{+}$ Tregs in *Hp+* patients with atrophy compared to those without, but no significant difference in Treg levels between patients with and without intestinal metaplasia (Hussain, 2012). In mouse models Tregs seem to protect from preneoplasia. TLR2 activated B cells suppressed *Hp*-induced preneoplastic changes by inducing IL-10 secreting Tr1 Tregs in the *H. felis* model (Sayi et al., 2011). Mice infected with *Hp* during the neonatal period were protected from preneoplasia compared to mice infected at 5-6 weeks of age and this protection was lost in TGF-$\beta^{-/-}$ mice (Arnold et al., 2011b).

6.2 AIMS

i. To compare T cell frequencies in *Hp*-infected and uninfected gastric mucosa.

ii. To determine which of these cell types are IL-17 producers.

iii. To correlate Th17 levels with virulence factor status of patients' *Hp* isolates.

iv. To correlate Th17 levels with histopathological findings and incidence of peptic ulcer disease and precancerous changes.

6.3 RESULTS

6.3.1 Relative Frequencies of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T Cells in the *Hp*-Infected Gastric Mucosa Compared to Uninfected Donors

Hp infection leads to a marked T cell infiltration of both the gastric epithelium and lamina propria (Bamford et al., 1998, Itoh et al., 1999). In keeping with this absolute T cell numbers, calculated using the microscopy mononuclear cell count, were higher in the biopsies from *Hp*-infected patients, though this did not reach significance (p=0.13)(Figure 6.2)

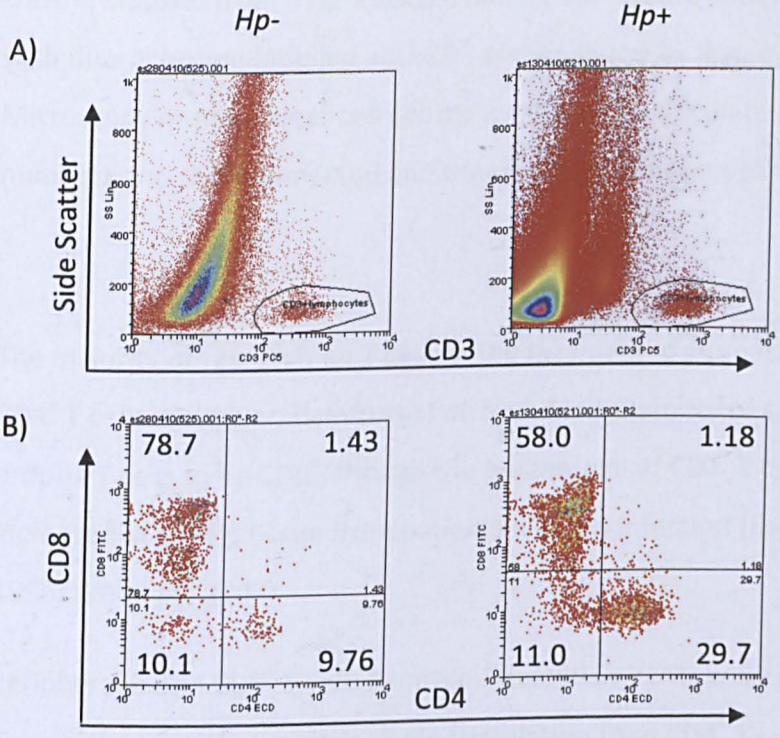


Figure 6.1 Example plots showing A) CD3 gating and B) $CD4^+$ and $CD8^+$ cell staining of gastric biopsy cells from uninfected and *Hp*-infected donors. 6 gastric biopsies from each patient were collected into culture medium, rubbed through a disposable cell strainer, washed, resuspended in culture medium, incubated overnight, then stained with the fluorochrome-labelled antibodies CD3-PC5, CD4-ECD and CD8-FITC and analyzed on a flow cytometer. Plots A) are ungated, plots B) are CD3 gated.

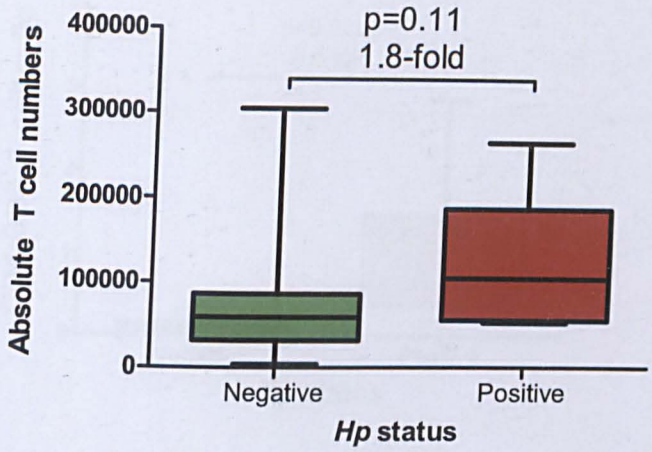


Figure 6.2 Trend towards increased T cells in *Hp*-infected gastric biopsies. Gastric biopsies from 8 *Hp*-infected and 11 uninfected patients were stained with fluorochrome-labelled anti-CD3 and analyzed by flow cytometry. Microscopy mononuclear cell counts were used to calculate absolute T cell numbers and the *Hp*-infected and uninfected groups compared.

The majority of research on T cells in the *Hp*-infected stomach has focused on $CD4^+$ T cells. However, Bamford *et al.* found the majority of gastric lamina propria T cells to be $CD8^+$, though the percentage of $CD4^+$ T cells was increased in gastric tissue from patients with *Hp* infection (Bamford et al., 1998, Wu et al., 2007).

Peripheral blood $CD8^+$ T cells from *Hp*-infected patients were reported to have a greater IFNγ response to *Hp* stimulation than $CD4^+$ T cells from the same patients, so $CD8^+$ T cells may contribute significantly to T cell cytokine production (Quiding-Jarbrink et al., 2001b).

Proportions of $CD4^+$ and $CD8^+$ T cells in unstimulated gastric biopsies from *Hp*-infected and uninfected patients were compared. Examples of the T cell gating strategy are shown in Figure 6.1A.

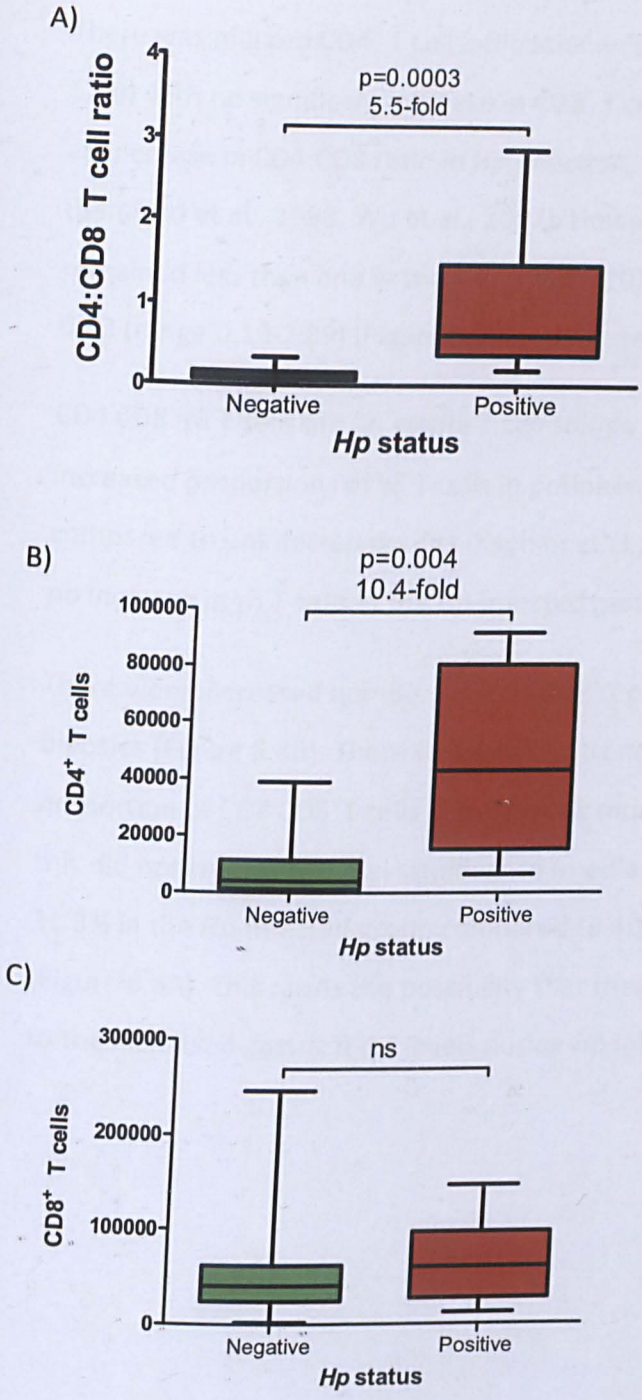


Figure 6.3 CD4 infiltration leads to an increased CD4:CD8 T cell ratio in the *Hp*-infected gastric mucosa. Gastric biopsies from 10 *Hp*-infected and 11 uninfected patients were stained with fluorochrome-labelled antibodies and analyzed by flow cytometry. Absolute $CD4^+$ and $CD8^+$ T cell counts were calculated using microscopy mononuclear cell counts and $CD4^+$ T cell, $CD8^+$ T cells and CD4:CD8 ratios in the infected and uninfected groups were compared.

There was marked $CD4^+$ T cell infiltration in the *Hp*-infected biopsies (Figure 6.3B) with no significant increase in $CD8^+$ T cells (Figure 6.3C). This resulted in an increase in CD4:CD8 ratio in *Hp* infection, consistent with previous reports (Bamford et al., 1998, Wu et al., 2007). However, the median CD4:CD8 ratio remained less than one in the majority (7/10) of the *Hp*+ samples, median 0.63 (range 0.19-2.89) (Figure 6.1B and Figure 6.3A).

$CD4^-CD8^-$ γδ T cells are an innate T cell source of IL-17. Kayhan *et al.* reported increased proportions of γδ T cells in peripheral blood of *Hp*-infected compared to uninfected donors (Kayhan et al., 2008), but Hatz *et al.* reported no increase in γδ T cells in the *Hp*-infected gastric mucosa.

There were increased numbers of $CD4^-CD8^-$ T cells in *Hp*-infected gastric biopsies (Figure 6.4B). There was a strong trend towards an increased proportion of $CD4^-CD8^-$ T cells in the gastric mucosa in *Hp* infection, although this did not reach statistical significance (median % $CD4^-CD8^-$ of $CD3^+$ cells was 11.3% in the *Hp*-infected group compared to 4.1% in the uninfected controls) (Figure 6.4A). This raises the possibility that these cells could be contributing to the increased gastric IL-17 levels during *Hp* infection.

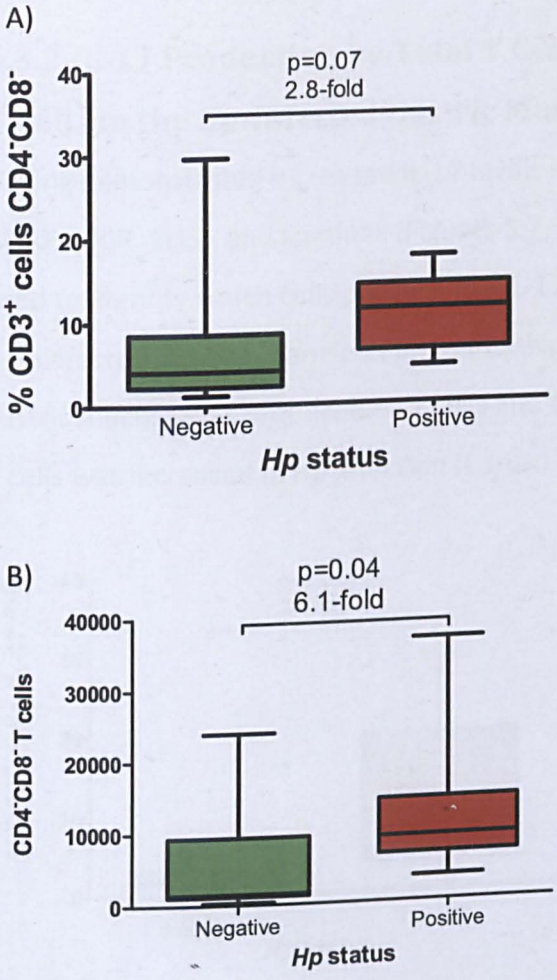


Figure 6.4 Increased $CD4^-CD8^-$ T cells in the *Hp*-infected gastric mucosa.

Gastric biopsies from 10 *Hp*-infected and 11 uninfected patients were stained with fluorochrome-labelled antibodies and analyzed by flow cytometry.

A) The percentage of $CD3^+$ cells that were $CD4^-$ and $CD8^-$ and B) Absolute $CD4^-$ $CD8^-$ T cell counts calculated using microscopy mononuclear cell counts in the infected and uninfected groups were compared.

6.3.2 IL-17 Production by Total T Cells, $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T Cells in the *Hp*-Infected Gastric Mucosa

Having demonstrated increased IL-17 levels in the *Hp*-infected gastric mucosa by RT-qPCR, ELISA and Luminex (Figures 5.2, 5.3 and 5.5) flow cytometry was used to identify which cells produce the IL-17. In a small study with 5 *Hp*+ and 5 uninfected patients Caruso *et al.* found that the vast majority of IL-17 in the gastric mucosa was produced by T cells and the percentage of IL-17-producing T cells was increased in *Hp* infection (Caruso et al., 2008).

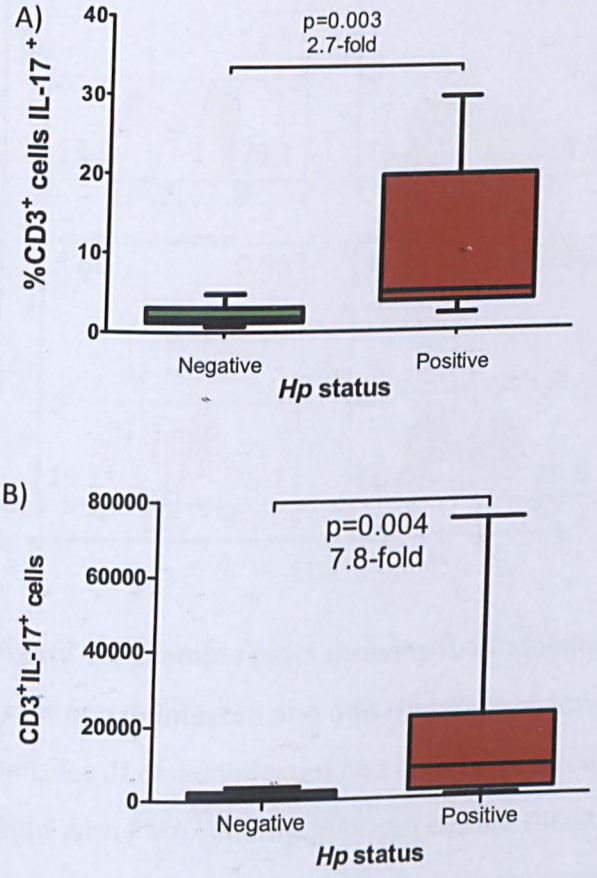


Figure 6.5 $IL\text{-}17^+$ T cells were increased in the *Hp*-infected gastric mucosa. Gastric biopsies from 10 *Hp*-infected and 11 uninfected patients were stained with fluorochrome-labelled antibodies and analyzed by flow cytometry. A) The frequency of $CD3^+IL\text{-}17^+$ events among the total $CD3^+$ population and B) $CD3^+IL\text{-}17^+$ cell counts calculated using microscopy mononuclear cell counts were compared between infected and uninfected patients.

This study confirmed an increase in both the percentage of IL-17$^+$ T cells and absolute numbers of IL-17$^+$ T cells in the *Hp*-infected stomach (Figure 6.5A and B). Staining with antibodies against CD4 and CD8 was used to further characterize the IL-17-producing T cells.

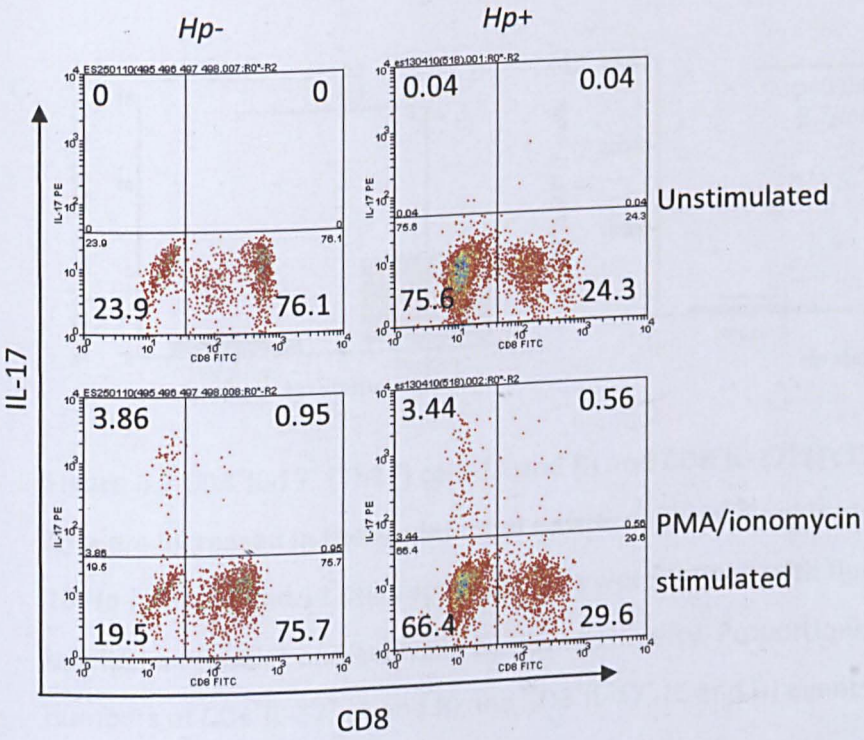


Figure 6.6 Example plots showing IL-17 staining of T cells in gastric CD3$^+$ cells from one uninfected and one *Hp*-infected donor. Cells extracted from gastric biopsies of one uninfected and one *Hp*-infected donor were incubated over night with PMA/ionomycin or cell culture medium only (unstimulated) and brefeldin A to block cytokine secretion, then stained with the fluorochrome-labelled antibodies CD3-PC5, CD8-FITC and IL-17-PE and analyzed by flow cytometry.

Examples of intracellular IL-17 staining are shown in Figure 6.6. Both CD4$^+$ and CD8$^+$ T cells were positively identified in this study. An unstimulated as well as a PMA/ionomycin-stimulated sample was analyzed for each patient.

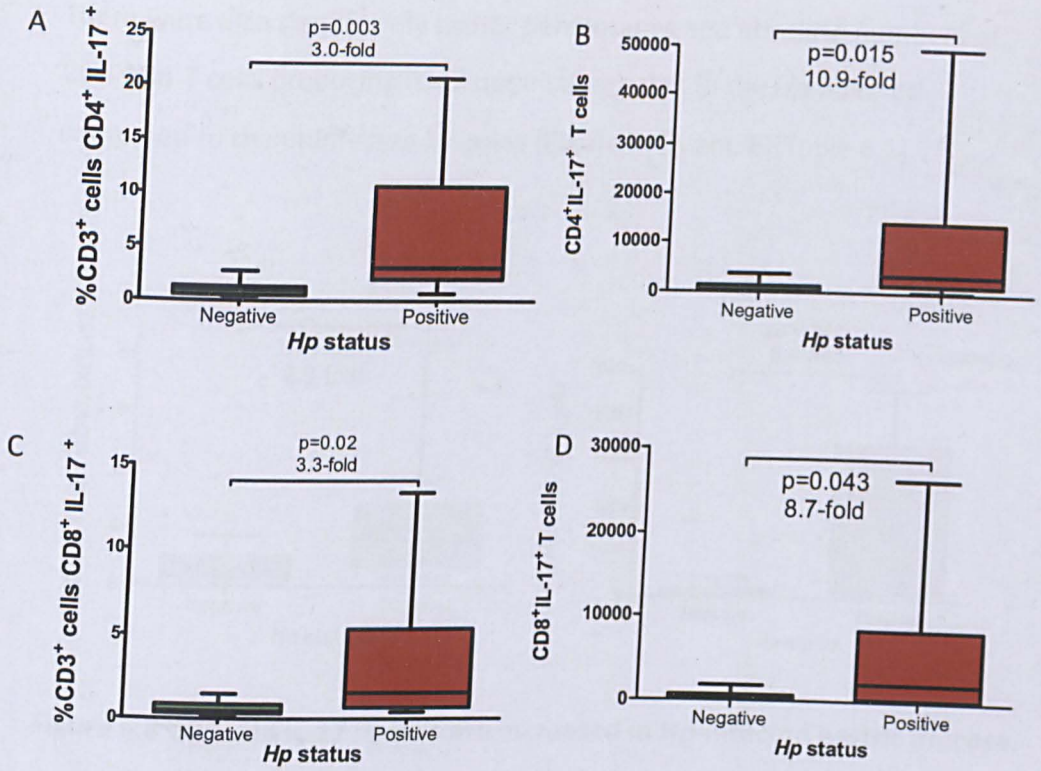


Figure 6.7 $CD4^+IL\text{-}17^+$ (Th17) cells (A and B) and $CD8^+IL\text{-}17^+$ (Tc17) cells (C and D) were increased in the *Hp*-infected gastric mucosa. Gastric biopsies from 10 *Hp*-infected and 11 uninfected patients were stained with fluorochrome-labelled antibodies and analyzed by flow cytometry. Proportions and absolute numbers of $CD4^+IL\text{-}17^+$ (A and B) and $CD8^+IL\text{-}17^+$ (C and D) events in the infected and uninfected groups were compared.

The percentages of $IL\text{-}17^+CD4^+$ T cells (Th17 cells) and $CD8^+$ T cells (Tc17 cells) were both increased in the *Hp*-infected group (Figure 6.7 A and C and Table 6.1). Absolute numbers of Th17 and Tc17 cells were also increased, with greater fold differences (Figure 6.7B and D).

There were also significantly higher percentages and absolute numbers of $CD4^-CD8^-$ T cells producing IL-17 upon stimulation in the *Hp*-infected compared to the uninfected biopsies (Figure 6.8A and B)(Table 6.1).

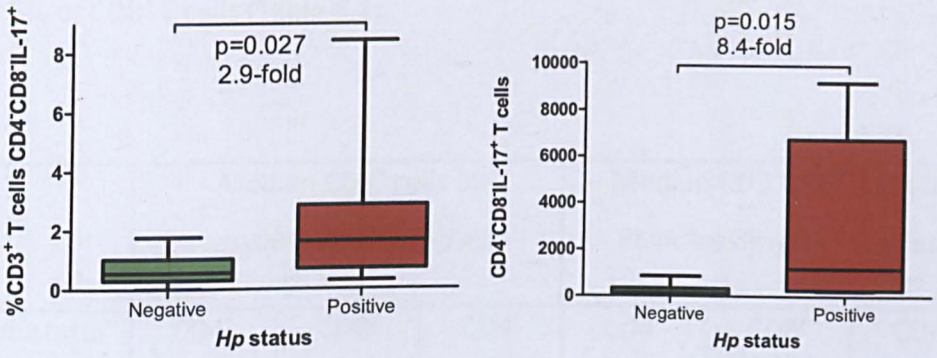


Figure 6.8 $CD4^-CD8^-IL\text{-}17^+$ T cells are increased in *Hp*-infected gastric mucosa. Gastric biopsies from 10 *Hp*-infected and 11 uninfected patients were stained with fluorochrome-labelled antibodies and analyzed by flow cytometry. A) Proportions of $CD4^-CD8^-IL\text{-}17^+$ T cells and B) Absolute numbers of $CD4^-CD8^-IL\text{-}17^+$ T cells in the infected and uninfected groups were compared.

The $CD4^-CD8^-$ T cells were a minority of the IL-17-secreting T cell population. $CD4^+$ T cells accounted for 68.5%, $CD8^+$ T cells 24.7% and $CD4^-CD8^-$ 18.2% of IL-17 producing T cells in the *Hp*+ biopsies (median percentages). However, relatively high proportions of the $CD4^-CD8^-$ T cells present in *Hp*-infected gastric biopsies secreted IL-17: 13.1%, compared to 8.0% of $CD4^+$ T cells and 3.5% of $CD8^+$ T cells (Table 6.1).

	Median $CD3^+$ cells in unstimulated samples			Median $CD3^+IL\text{-}17^+$ cells in PMA/ionomycin samples		
Hp status of patients	$CD4^+$	$CD8^+$	$CD4^-$ $CD8^-$	$CD4^+$	$CD8^+$	$CD4^-$ $CD8^-$
Negative	4134	40997	1471	314	240	141
Positive	43193	60279	9016	3434	2101	1181
p value	p=0.004	Ns	p=0.035	p=0.015	p=0.043	p=0.015

Table 6.1 Summary of median numbers of the T cell subsets in unstimulated samples, and in IL-17 secreting T cell populations in PMA/ionomycin stimulated samples, in 8 *Hp*+ and 11 uninfected patients. Mann-Whitney-U test was used to compare *Hp*-infected and uninfected groups and the resulting p values are given.

6.3.3 Expression of Th17 Transcription Factor *RORC2* in *Hp* Infection and Correlation of Th17 Markers

RORγt was identified as the transcription factor that directs Th17 differentiation in mice by Ivanov *et al.* in 2006 (Ivanov et al., 2006). The human orthologue, RORC2, is generally accepted as the Th17 lineage-defining transcription factor and one of the most specific Th17 markers (Crome et al., 2009). However, it may also be expressed in $CD8^+$ T cells and innate cells such as NKT cells (Burgler et al., 2009). RORγt expression has been reported in IL-17-producing γδ T cells, lymphoid tissue inducer-like cells and other IL-17-producing innate lymphoid cells (ILC3s) in mice (Sutton et al., 2009, Takatori et al., 2009, Buonocore et al., 2010).

RORC2 expression in gastric biopsies from *Hp*-infected and uninfected patients was quantified in this study as a measure of IL-17-producing cells.

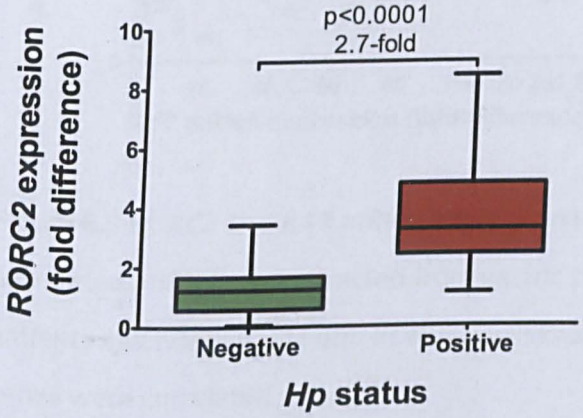


Figure 6.8 The Th17 transcription factor, *RORC2* is increased in the *Hp*-infected gastric mucosa. mRNA was extracted from gastric tissue biopsies from 41 *Hp*+ and 15 uninfected patients and relative *RORC2* levels calculated by normalizing against *GAPDH* and expressing levels relative to a negative comparator reference sample.

As demonstrated in the previous section, the majority of IL-17-producing T cells in the *Hp*-infected gastric mucosa are Th17 cells, though Tc17 and $CD4^-$ $CD8^-$ T cells also make a contribution.

RORC2 expression was increased in *Hp*-infected gastric mucosal tissue (Figure 6.8). This is in keeping with the highly significant increased in *IL17* expression found in *Hp*-infected gastric tissue (Figure 5.2), though the fold difference in *IL17* expression was much greater than that found for RORC2 expression (42.6-fold vs 2.7-fold). There was a trend towards correlation of *RORC2* with *IL17* mRNA levels but this did not reach statistical significance (Figure 6.9).

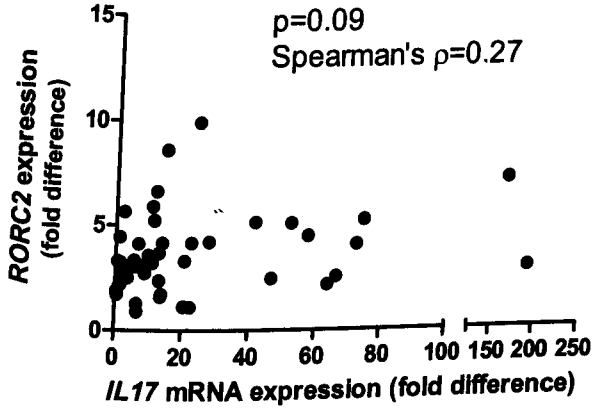


Figure 6.9 *RORC2* and *IL17* mRNA expression were not significantly correlated. mRNA was extracted from gastric tissue biopsies from 41 *Hp*+ patients and relative *IL17* and *RORC2* levels calculated. Values for the two genes were correlated.

Although *IL17* mRNA and IL-17 protein levels correlated (Figure 5.5B), no correlation was found between *RORC2* mRNA expression and IL-17 levels measured by Luminex (data not shown). RORC2 was not measured at the protein level in this study.

There were insufficient *Hp*+ patients with both Th17 frequencies by flow cytometry and *RORC2* mRNA expression, *IL17* mRNA or IL-17 Luminex measurements to correlate these variables.

6.3.4 Correlation of *RORC2* Expression with Virulence Factor Status

The *Hp* virulence factors cagA (marker for the *cagPAI*) and dupA are associated with increased risk of disease (sections 1.1.5.1 and 1.1.5.3). Mutation of both these virulence factors leads to lower production of IL-23 by MoDCs stimulated with *AB21 Hp* (Figure 3.9A). This raises the possibility that these virulence factors could be associated with higher levels of the Th17-differentiating cytokine, IL-23, *in vivo.*

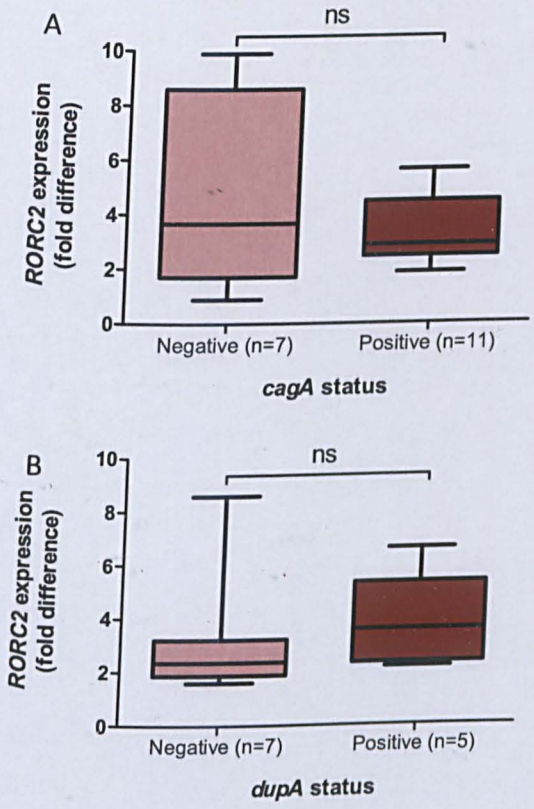


Figure 6.10 Association of *RORC2* expression with expression of the virulence factor *cagA* and *dupA* genes. *RORC2* mRNA expression in *Hp*+ biopsies (normalized against *GAPDH* and expressed relative to a negative comparator reference sample) was stratified by A) *cagA* status and B) *dupA* status.

Patients' *RORC2* mRNA expression levels were therefore stratified by *cagA* and *dupA* status of their infecting strains.

No significant difference was found in *RORC2* mRNA expression between patients infected with *Hp* strains with or without the *cagA* or *dupA* virulence factor genes. Numbers in the some of the groups were low but these findings are consistent with the lack of difference detected in IL-17 levels in patients with and without these virulence factors in Chapter 5 (Figure 5.12A and data not shown).

6.3.5 Correlation of *RORC2* Expression with Histopathology and Disease Status

As Th17 cells are known to be important for recruitment of lymphocytes and neutrophils and IL-17 levels correlated with inflammation scores (measure of lymphocyte infiltration) and activity scores (measure of neutrophil infiltration) (section 5.3.6), *RORC2* expression was also stratified by inflammation and activity scores.

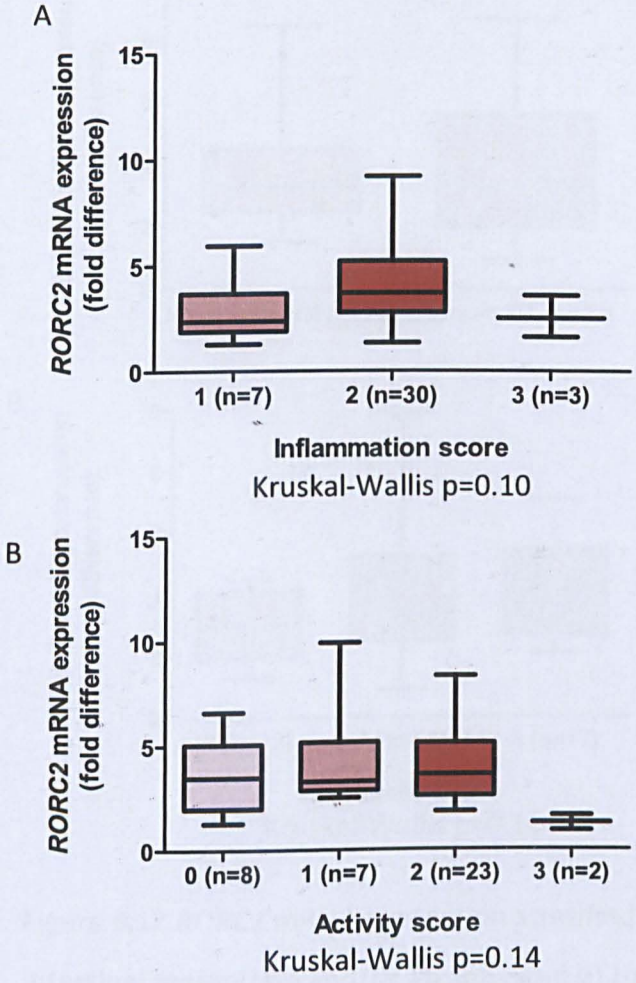


Figure 6.11 Association of *RORC2* mRNA expression with inflammation and activity scores. *RORC2* expression in biopsies from 40 *Hp*+ patients (normalized against *GAPDH* and expressed relative to a negative comparator reference sample) were stratified by A) Inflammation score (lymphocyte infiltration) and B) Activity score (neutrophil infiltration), scored by an experienced gastrointestinal histopathologist.

No significant association between *RORC2* expression and inflammation or activity scores was found (Figure 6.11A and B).

Th17 cells are increased in a number of cancers (section 1.3.6) so next precancerous changes were correlated with *RORC2* mRNA expression to help address the hypothesis that Th17 cells may contribute to the development of cancer in the inflamed stomach.

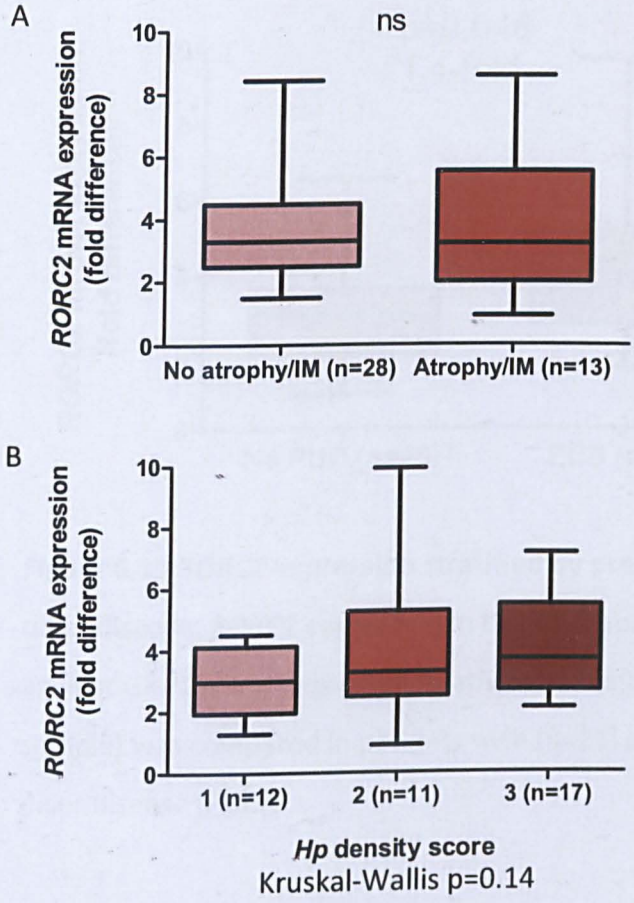


Figure 6.12 *RORC2* mRNA expression stratified by A) Presence or absence of intestinal metaplasia and/or atrophy and B) *Hp* density score. *RORC2* expression in biopsies from 40/41 *Hp*+ patients (normalized against *GAPDH* and expressed relative to a negative comparator reference sample) were stratified for A) Atrophy and/or intestinal metaplasia and B) *Hp* density, scored by an experienced gastrointestinal histopathologist. ns= no significant difference (Mann-Whitney U-test).

As for IL-17 (Chapter 5, Figure 5.14A), there was no association between *RORC2* expression and the presence or absence of precancerous changes (Figure 6.12A). Median *RORC2* expression levels increased with increasing *Hp* density score, but there was no statistically significant association between these parameters (Figure 6.12B).

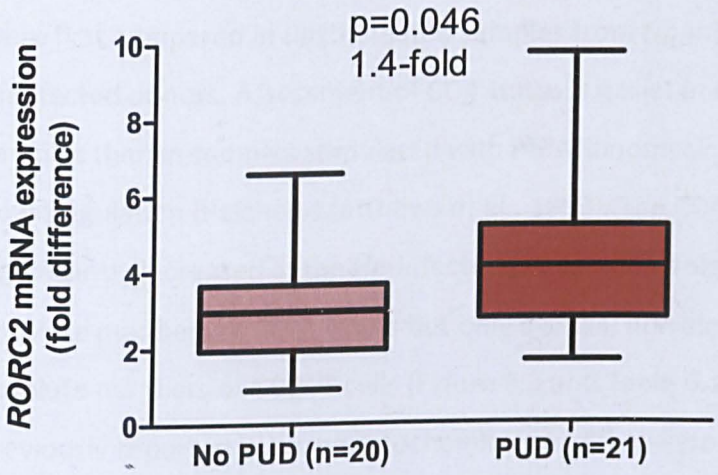


Figure 6.13 *RORC2* expression stratified by presence or absence of peptic ulcer disease. *RORC2* expression in biopsies from 41 *Hp*+ patients (normalized against *GAPDH* and expressed relative to a negative comparator reference sample) was compared in patients with (n=21) and without (n=20) peptic ulcer disease (PUD).

Despite finding no significant correlation between *RORC2* mRNA expression and histopathological findings (Figures 6.11 and 6.12) or between IL-17 and peptic ulcer disease (Figure 5.15), peptic ulcer disease appeared to be associated with significantly higher levels of *RORC2* expression. This finding needs to be taken with some caution as the difference in *RORC2* expression between the peptic ulcer disease and ulcer-free groups was only 1.4-fold. This may suggest that Th17 cytokines other than IL-17, or a combination of factors are required for the development of peptic ulceration (see discussion).

6.4 DISCUSSION

6.4.1 Frequencies of the Different T Cell Subtypes in the *Hp*-Infected Gastric Mucosa and their Production of IL-17 upon Stimulation.

Caruso *et al.* reported that T cells are the main source of gastric IL-17 in *Hp* infection (Caruso et al., 2008). To assess which T cell subgroups may be significant contributors of IL-17, frequencies of the different T cell subtypes were first compared in unstimulated samples from *Hp*-infected and uninfected donors. Assessment of CD4 status is easier in unstimulated samples than in samples stimulated with PMA/ionomycin, as PMA causes CD4 downregulation (Pelchen-Matthews et al., 1993). The CD4:CD8 ratio was significantly increased in the *Hp*-infected group, with a significant increase in absolute numbers of $CD4^+$ T cells but only a small, non-significant increase in absolute numbers of $CD8^+$ T cells (Figure 6.3 and Table 6.1), consistent with previously reported immunohistochemistry and flow cytometry findings (Bamford et al., 1998, Wu et al., 2007). However, $CD8^+$ T cell numbers were high in uninfected patients and the median $CD8^+$ T cell count remained higher that the median $CD4^+$ T cell count in the *Hp*-infected donors (Table 6.1). Some of the CD8 cells identified may be CD8α:α T cells. "Unconventional" T cells that do not have an α:β TCR account for a significant proportion of mucosal T cells in the gastrointestinal tract (Janeway et al., 2005).

A number of innate sources of IL-17 have recently been described, including $CD3^+CD4^-CD8^-$ γδ T cells (section 1.3.9)(Sutton et al., 2009). Frequencies of $CD3^+CD4^-CD8^-$ T cells in *Hp*-infected and uninfected gastric biopsies were therefore compared. $CD3^+CD4^-CD8^-$ T cell numbers were increased in the *Hp*-infected group, though the increase in the proportion of T cells not expressing CD4 or CD8 did not reach statistical significance (Figure 6.4A and B). The $CD3^+CD4^-CD8^-$ T cells were a minority population (median 8.0% of T cells in the *Hp*+ group). Hatz *et al.* previously found no significant increase in γδ T cells in *Hp*-infected lamina propria using immunohistochemistry (Hatz et al., 1996).

Futagami *et al.* found increased γδ T cells only in the *Hp*-infected group with the most severe gastritis (Futagami et al., 2006). They also noted significantly higher γδ T cells in those with lymphoid follicles (Futagami et al., 2006).

The method used in this study investigates epithelial lymphocytes as well as lamina propria lymphocytes, though in a number of flow cytometry studies, including the studies by Hatz *et al.* and Futagami *et al.* the epithelium has been removed in order to study lamina propria lymphocytes only (Hatz et al., 1996, Bamford et al., 1998, Lundgren et al., 2005). The epithelium may be the source of many of the $CD3^+CD4^-CD8^-$ T cells. Epithelial lymphocytes could be an important source of cytokines *in vivo.* However, Trejdosiewicz *et al.* found no increase in intraepithelial γδ T cells in *Hp* infection (Trejdosiewicz et al., 1991).

$CD4^+$IL-17^+ Th17 and $CD8^+$IL-17^+ Tc17 cells were both significantly increased in *Hp* infection (approximately 3-fold), with median Th17 numbers 1.6-fold higher than median Tc17 numbers. This suggests that Th17 cells are the conventional T cell population that contributes the most IL-17 during *Hp* infection, though the relative amounts of IL-17 produced by the different populations were not compared in this study. A lower proportion of the $CD8^+$ T cells present are IL-17-producers, but $CD8^+$ T cells are present at high frequencies in the gastric mucosa of many *Hp*-infected patients and are a significant source of IL-17.

A relatively high proportion of $CD3^+CD4^-CD8^-$ T cells produced IL-17, so this population made a significant IL-17 contribution despite being present in relatively low numbers (Table 6.1). It will be important to further characterize and positively identify these cells in future studies but they are likely to represent innate T lymphocytes, which could include γδ T cells and NKT cells. Using flow cytometry O'Keefe *et al.* found NKT cells accounted for <2% T cells in uninfected gastric mucosa, with no significant increase in *Hp* infection, suggesting that other innate T cells are involved (O'Keeffe et al., 2008).

These innate lymphocytes are likely to be important sources of cytokine early in the immune response, before conventional adaptive T cell responses have had time to develop. *Hp* can directly stimulate γδ T cells to produce IFNγ and TNF-α in the absence of antigen presenting cells (Romi et al., 2011). IL-23 and IL-1β can stimulate γδ T cells to produce IL-17 without a requirement for TCR engagement (Sutton et al., 2009). Mouse $CD4^+$ T cells express IL-17R and γδ T cell-derived IL-17 can boost IL-17 production by $CD4^+$ T cells (Sutton et al., 2009). This suggests that the IL-17 produced by γδ T cells and other innate T lymphocytes may have an important role in boosting conventional Th17 cell IL-17 production.

6.4.2 Expression of Th17 Transcription Factor *RORC2* in *Hp* Infection and Correlation with IL-17

RORC2 is generally accepted as the Th17 lineage transcription factor and one of the best Th17 markers. However expression of *RORC2* or the mouse equivalent *RORγt* has been described in other IL-17-producing cells, including $CD8^+$ T cells, NKT cells, γδ T cells, LTi-like cells and other ILC3s (section 1.3.9)(Takatori et al., 2009, Burgler et al., 2009, Sutton et al., 2009, Buonocore et al., 2010). This is the first study that I am aware of to investigate *RORC2* mRNA expression in the *Hp*-infected human gastric mucosa. As expected, in view of the marked increase in IL-17 during *Hp* infection, *RORC2* expression was also significantly increased (Figure 6.8).

There was a strong trend towards correlation between *RORC2* and *IL17* mRNA expression, but this did not reach statistical significance (Figure 6.9). *RORC2*-expressing cells may represent a variety of types of IL-17-producing cells with heterogeneous IL-17 expression.

Ideally *RORC2* expression would have also been studied at the protein level but limited reagents were available. Flow cytometry was attempted with a RORC2-PE antibody but problems with non-specific staining made this uninformative, despite attempts at optimization with lower concentrations of antibody and FcR blocking.

6.4.3 Correlation of *RORC2* mRNA Expression with Virulence Factor Status

No correlation was found between *RORC2* expression and *cagA* or *dupA* status of the infecting strains. However, numbers analyzed were small, so a link cannot be completely excluded.

There was a weak trend for higher *RORC2* mRNA expression in the biopsies infected with strains thought to have functional *dupA1*, forms of dupA, as described by Hussein *et al.* (Hussein et al., 2010). The mode of action of dupA and the exact genes required for its function are not completely understood, though this virulence factor appears to have effects on immune cells including monocytes and dendritic cells (Hussein et al., 2010)(Figure 3.9). IL-17 levels were also higher, though not significantly so, in the *dupA*+ group (data not shown). It would be interesting to explore this further in a larger number of *Hp*-infected biopsies.

6.4.4 Correlation of *RORC2* mRNA Expression with Histopathology and Disease Status

There were trends for correlations between *RORC2* mRNA expression and inflammation and activity scores, but these did not reach statistical significance (Figure 6.11A and B). These trends were not as strong as those between IL-17 protein levels and inflammation and activity scores shown in Chapter 5 (Figure 5.13A and B). *RORC2* expression is perhaps a less direct measure, as it assumes that mRNA levels are reflected at the protein level and that RORC2 in turn reflects Th17 activity.

Though Th17 levels are known to be increased in gastric cancer and a number of other malignancies, as discussed in the introduction, no difference in *RORC2* expression was found between those with precancerous changes and those without (Figure 6.12A). This is consistent with lack of difference in IL-17 levels between these groups (Chapter 5). This does not support a critical role for Th17 cells in carcinogenesis in the *Hp*-infected stomach, but it is possible

that a small effect would be found in a larger study. IL-17/Th17 may have a role in progression of established gastric cancer.

There was a trend for correlation of *RORC2* expression with *Hp* density score (Figure 6.12B). This is consistent with the increased Th17 response in *Hp+* patients demonstrated with flow cytometry. The Sydney scoring system is category based rather than quantitative, which limits the statistical power for finding correlations. Quantitative scoring of inflammation, activity and *Hp* density may help confirm if IL-17 and *RORC2* expression are indeed associated with these variables.

Patients with peptic ulcer disease had significantly higher *RORC2* expression than those without (Figure 6.13). However no difference in IL-17 levels between the ulcer and ulcer-free groups was detected (Figure 5.15). This finding needs to be taken with some caution as the difference in *RORC2* levels was small and only just statistically significant. It is possible that small differences in IL-17 levels in patients with and without peptic ulcer disease may be detected in a larger study. However these findings raise the possibility that Th17 cells may have pathogenic effects in the stomach by a mechanism other than secretion of IL-17.

GM-CSF is produced by some Th17 cells and was shown to be essential for pathogenicity in EAE (Codarri et al., 2011, El-Behi et al., 2011). However, GM-CSF levels measured by Luminex did not differ between 32 *Hp+* and 19 uninfected patients, or in the *Hp+* patients with and without ulceration.

Pro-inflammatory Th17 cells in inflamed tissue often produce IFNγ in addition to IL-17. These "double-producers" were initially described in the inflamed bowel of patients with Crohn's disease but have since been described by many authors both *in vivo* and *in vitro* (section 1.3.3 and 4.4.2)(Volpe et al., 2008, Annunziato et al., 2007, Abromson-Leeman et al., 2009, Ghoreschi et al., 2010, Kebir et al., 2009). Their differentiation seems to be favoured by a lack of TGF-β, which allows T-bet expression (Ghoreschi et al., 2010). Their phenotype is more similar to Th17 cells than Th1 cells, even if they lose IL-17

expression (Boniface et al., 2010). IFNγ is strongly associated with gastric inflammation in human and animal studies, as discussed in Chapter 5. Patients with peptic ulcer disease tended to have more gastric $CD4^+IFN\gamma^+$ cells (Robinson et al., 2008). IFNγ is upregulated less than IL-17 during *Hp* infection (section 5.3.4), but may still have significant pathogenic effects. It may be partly produced by Th17 cells and may have potent effects, even at relatively low levels, in synergy with IL-17.

The balance between Tregs and Th17 cells in the *Hp*-infected gastric mucosa is likely to influence risk of disease. These lineages are closely related (section 1.3.2 and 1.3.3). IL-17-producing human $FOXP3^+$ T regs have been described (Voo et al., 2009). $FOXP3^+ROR\gamma t^+IL\text{-}10^+$ cells were found to be in equilibrium with $ROR\gamma t^+IL\text{-}17^+$ cells in mice, with FOXP3 and CCL20 favouring IL-10 production and IL-6 and IL-23 favouring IL-17 production (Lochner et al., 2008). *Staphylococcus aureus*-primed Th17 cells had the potential to produce IL-10 and IL-1β blockade led to increased IL-10 production (Zielinski et al., 2012). IL-27 promotes the development of IL-10 secreting Tr1 Tregs, whilst inhibiting Th17 development, but has little effect on committed Th17 cells (Pot et al., 2011, El-behi et al., 2009).

Overall the gastric cytokine findings in Chapter 5 indicate that CCL20 and IL-27 are significantly increased in the *Hp*-infected gastric mucosa (Figures 5.7A and 5.9B), whereas IL-1β, IL-6, and IL-23 are not. This might be expected to lead to predominance of regulatory IL-10 (from Tregs and possibly also Th17 cells) over pro-inflammatory IL-17. This is consistent with the absence of clinical disease in the majority of *Hp*-infected patients. It is likely that the balance of cytokines is altered in those with *Hp*-induced pathology but the relative importance and potency of the various cytokines is not yet clear.

In summary IL-17 in the *Hp*-infected gastric mucosa is produced mainly by Th17 cells, though Tc17 and $CD4^-CD8^-$ T cells also make a significant contribution. Expression of the Th17 transcription factor *RORC2* is also increased in *Hp*-infected gastric mucosa and high *RORC2* mRNA expression is

associated with peptic ulcer disease, though IL-17 levels were not. IL-17 was associated with high activity and inflammation scores. No associations between *RORC2* mRNA expression or IL-17 levels and *cagA* or *dupA* virulence factor status of the infecting strains or presence or absence of precancerous changes were detected.

CHAPTER 7

DISCUSSION

7. DISCUSSION

7.1 MAIN FINDINGS AND THEIR SIGNIFICANCE

7.1.1 T Helper Cell Differentiation in *Hp* Infection

7.1.1.1 Hp stimulation of MoDCs – relative IL-12p70 and IL-23 secretion and effects of mutating cagA and dupA virulence factors

Most previous studies have used MoDCs to investigate DC responses to *Hp* (Guiney et al., 2003, Kranzer et al., 2004, Kranzer et al., 2005, Hafsi et al., 2004, Mitchell et al., 2007, Khamri et al., 2010, Andres et al., 2011). In this study relative IL-12p70 and IL-23 production by *Hp*-stimulated DCs were compared using two different DC models and the effects of mutating the cagA and dupA virulence factors were investigated (Chapter 3).

Hp stimulation of MoDCs resulted in production of a broad range of inflammatory cytokines (IL-12p40, IL-12p70, IL-8, IL-6, IL-23 and IL-1β) and the anti-inflammatory IL-10, strong upregulation of activation markers and high IFNγ production when the *Hp*-stimulated MoDCs or their supernatants were cultured with naïve T cells, consistent with previously published data (Bimczok et al., 2010, Hafsi et al., 2004, Guiney et al., 2003, Kranzer et al., 2004, Khamri et al., 2010). Relative IL-12p70 and IL-23 levels produced by *Hp*-stimulated DCs have not been investigated previously. IL-12p70 levels produced by *Hp*-stimulated MoDCs were 12-fold and 15-fold higher than IL-23 levels for the AB21 and AB31 *Hp* strains. This was reflected at the T cell level with high IFNγ and little IL-17 production when *Hp*-stimulated MoDCs were cultured with naïve T cells, suggesting a strong Th1 bias.

Stimulation with isogenic *dupA* and *cagA AB21 Hp* mutants led to lower IL-12p70 and IL-23 responses than stimulation with the wild-type strains. The effect of *dupA* mutation on MoDC cytokine secretion was less marked than the effect found on monocytes, but monocytes are not present in the gastric mucosa (Hussein et al., 2010).

7.1.1.2 Comparison of Hp stimulation of MoDCs and MyDCs

Circulating $CD1c^+$ MyDCs were also studied and found to produce lower IL-23 concentrations than the MoDCs, and no detectable IFNγ following *Hp* stimulation. Naïve T cells cultured with *Hp*-stimulated MyDCs produced similar IL-17 levels to those cultured with *Hp*-stimulated MoDCs and secreted significant concentrations of IFNγ despite the lack of IL-12p70. Bimczok *et al.* studied gastric DCs from patients having gastric resections for obesity. Similar to the current findings for *Hp*-stimulated MyDCs, they found that *Hp*-stimulated gastric DCs did not produce any IL-12p70 at the mRNA or protein level, however T cells cultured with the *Hp*-stimulated gastric DCs did produce IFNγ (Bimczok et al., 2010). This suggests that MyDCs might be a better model for gastric DCs than the more widely used MoDCs. Further studies by Bimczok *et al.* showed that gastric stromal factors reduced IL-12 and Th1 responses and inhibited upregulation of DC activation markers in response to *Hp* (Bimczok et al., 2011).

Luminex analysis of supernatants from homogenized snap frozen gastric biopsies showed much higher IL-23 than IL-12p70 levels, again more in keeping with the MyDC and gastric DC data than the MoDC data. This method measures cytokine content of the entire gastric biopsy and will therefore include cytokines produced by other cell types, but should give a good reflection of the cytokine milieu in the gastric mucosa.

The site of T cell priming by antigen presenting cells in human *Hp* infection is uncertain. In mouse models Peyer's patches in the small intestine seem to play an important role, but lymphoid follicles can also form in *Hp*-infected human gastric mucosa, and may contribute (Nagai et al., 2007, Kiriya et al., 2007). Regardless of the initial site of T cell priming, many $CD4^+$ T cell populations are known to have a degree of plasticity (Rowell and Wilson, 2009, Lee et al., 2009), so the gastric cytokine environment could influence $CD4^+$ T cell phenotype.

7.1.2 The Cytokine Milieu in the *Hp*-Infected Gastric Mucosa

7.1.2.1 Measurement of cytokines in snap frozen gastric biopsies

Luminex technology was used to measure relative levels of a broad panel of cytokines in supernatants from homogenized snap frozen gastric biopsies from *Hp*-infected and uninfected patients (Chapter 5). Endoscopic mucosal biopsies are small (typically approximately 5 mg) and levels of many of the cytokines of interest are low, so measuring multiple cytokines in a single sample represents a technical challenge. Other investigators have used semi-quantitative methods including immunohistochemistry (Lehmann et al., 2002, Lindholm et al., 1998, Holck et al., 2003) and western blotting (Luzza et al., 2000, Tomita et al., 2001), or PCR-based methods to quantitate cytokine mRNA, which may or may not be reflected as the protein level (Yamaoka et al., 2001, Luzza et al., 2001, Yamaoka et al., 1995, Serelli-Lee et al., 2012, Yamauchi et al., 2008). Cytokines have been measured in gastric biopsy homogenates using ELISA (Serelli-Lee et al., 2012, Shimizu et al., 2004, Yamaoka et al., 1997, Yamaoka et al., 2001, Yamauchi et al., 2008, Caruso et al., 2008), but additional volume (causing dilution) is required for each cytokine assayed, so the number of cytokines, particularly those present at low levels, that can be assayed using this method is limited. Another approach is to culture gastric biopsies *in vitro* and measure cytokines in the supernatants (Bodger et al., 1997, Mizuno et al., 2005, Crabtree et al., 1991). Cytokines in the supernatants of cultured lamina propria mononuclear cells and T cell clones have also been measured and intracellular cytokine staining can be assessed by flow cytometry (Luzza et al., 2000, Caruso et al., 2008, Lehmann et al., 2002, D'Elios et al., 1997, Bamford et al., 1998, Robinson et al., 2008). However, all these methods require *in vitro* culture with or without stimulation which can alter the cytokine profile (Veldhoen et al., 2009). The cytokine levels in homogenates of snap frozen gastric biopsies should more accurately reflect those found in the gastric mucosa *in vivo.*

7.1.2.2 Relative concentrations of T helper subset effector cytokines in the Hp-infected gastric mucosa

Levels of the Th17 cytokine IL-17 were significantly higher than the signature cytokines of the other main $CD4^+$ T cell subsets IFNγ (Th1), IL-4 (Th2) and IL-10 (Treg) in gastric biopsies from *Hp*-infected patients and significantly higher than found in uninfected biopsies. In keeping with this, levels of IL-17F and chemokines CCL20 and IL-8, which are targeted by IL-17, were also increased.

IL-21 and IL-22 can also be produced by Th17 cells, yet IL-21 levels were lower in *Hp*-infected gastric biopsies than those from uninfected controls and there was no significant difference between IL-22 levels in biopsies from the *Hp*-infected and uninfected groups. These IL-22 findings are consistent with ELISA and immunofluorescence microscopy published by Serelli-Lee *et al* (Serelli-Lee et al., 2012). IL-22 production by Th17 cells is dependent on availability of AhR ligands (Veldhoen et al., 2009) and can be suppressed by high levels of TGF-β (Rutz et al., 2011). Th1 and innate immune cells can also secrete IL-22, which could mask small changes in Th17-derived IL-22 in *Hp*-infected gastric mucosa.

The IL-21 findings are unexpected, as Caruso *et al.* found increased IL-21 in *Hp*-infected gastric biopsies at both protein and mRNA levels by western blotting and RT-qPCR (Caruso et al., 2007a). Further studies are required to clarify these discrepancies. IL-21 promotes $CD8^+$ T cell expansion, so high IL-21 levels in gastric biopsies from uninfected patients could be consistent with the higher proportion of $CD8^+$ cells found in these samples.

7.1.2.3 Concentrations of cytokines involved in Th17 differentiation in the Hp-infected gastric mucosa

IL-21 is also involved in early Th17 differentiation. No significant differences in levels of IL-1β and IL-6, also key in early Th17 differentiation, were found between the *Hp*-infected and uninfected groups. TGF-β levels were not measured in this study, but have been found to be increased in the *Hp*-infected gastric mucosa at the mRNA level and by immunohistochemistry

(Lindholm et al., 1998, Robinson et al., 2008, Li and Li, 2006, Harris et al., 2008, Kandulski et al., 2008). This suggests that gastric Th17 cells may be differentiated from naïve T cells at another site such as the Peyer's patches. Another alternative is that memory Th17 cells are recruited from the periphery. Consistent with this theory, high levels of CCL20 (chemokine ligand for $CCR6^+$ Th17 cells) are present in the gastric mucosa, and are increased in *Hp*-infected compared to uninfected biopsies.

IL-23 levels in the gastric mucosa are very high (though not found to be increased during *Hp* infection in this study). This cytokine is essential for terminal Th17 differentiation and effector function (McGeachy et al., 2009). In combination with other pro-inflammatory cytokines it also promotes $CD4^+IL\text{-}17^+IFN\gamma^+$ pathogenic populations (Wilson et al., 2007, Volpe et al., 2008, Ghoreschi et al., 2010). It is therefore likely to be key to maintaining the phenotype and function of Th17 cells in the gastric mucosa.

7.1.2.4 Concentrations of CCL20 and Tr1-differentiating IL-27 in the Hp-infected gastric mucosa

CCL20 can be produced by Th17 cells, but also attracts them due to their expression of CCR6 (Wilson et al., 2007, Wu et al., 2007). Tregs also express CCR6 and may be recruited to the gastric mucosa by the same mechanism. Levels of IL-27, which promotes IL-10-producing Tr1 Tregs at the expense of Th17 cells (Pot et al., 2011), are high within the gastric mucosa and increased in *Hp* infection. This raises the possibility that some *de novo* Treg differentiation may occur in the *Hp* infected stomach. IL-27 has little effect on mature Th17 cells (El-behi et al., 2009), in keeping with the strong Th17 response and high IL-17 levels in the *Hp*-infected gastric mucosa.

7.1.3 Peripheral Blood and Gastric Th17 Responses to *Hp*

7.1.3.1 IL-17-producing cells in the Hp-infected gastric mucosa

Having identified a dominant IL-17 response to *Hp* infection, flow cytometry studies were undertaken to identify which cells were the source of the IL-17 (Chapter 6). In *Hp* infection the gastric $CD4^+$ T cell numbers increased, whilst

there was no significant change in $CD8^+$ T cell numbers, but there were significant increases in both $CD4^+IL\text{-}17^+$ Th17 cells and $CD8^+IL\text{-}17^+$ Tc17 cells in the *Hp*-infected compared to uninfected gastric biopsies. There was also a significant increase in $CD4^-CD8^-$ T cells, and numbers of these cells producing IL-17 during *Hp* infection. These are likely to be innate T cells, possibly γδ T cells. Other unconventional T cells can also occur in the gut mucosa, including CD8α:α cells which may express the αβ or γδ TCR (Janeway et al., 2005). In uninfected tissue γδ T cells are largely restricted to the gastric epithelium (Hatz et al., 1996, Trejdosiewicz et al., 1991, Futagami et al., 2006), but γδ T cell infiltration of the lamina propria has been reported in *Hp*-associated gastritis (Futagami et al., 2006). In the current study whole gastric biopsies, including epithelium and lamina propria, were homogenized, which may explain the relatively high numbers of $CD4^-CD8^-$ T cells found. IL-17 produced by these cells may boost production from conventional αβ IL-17 producing cells such as Th17 cells (Sutton et al., 2009).

Expression of the Th17 transcription factor *RORC2* was upregulated during *Hp* infection, consistent with the flow cytometry findings. However, *RORC2* is not completely specific for Th17 cells, as it is also expressed by other IL-17 producing cells including $CD8^+$ T cells and NKT cells and RORγt expression has been reported in a number of types of innate IL-17 producing cells, including γδ T cells and the recently designated ILC3s including LTi cells (Burgler et al., 2009, Sutton et al., 2009, Takatori et al., 2009, Buonocore et al., 2010, Spits et al., 2013).

ILC3s are described in detail in section 1.3.9. These innate lymphoid cells express RORγt and produce IL-17 and/or IL-22 but are negative for the lineage markers CD3 and CD4 in humans (Spits et al., 2013). The flow cytometry analysis in this study was gated on CD3 and would therefore have excluded these cells, but they may have contributed to the total IL-17 and/or IL-22 measured in the gastric biopsies by RT-qPCR and Luminex. Caruso *et al.* found that the vast majority of $IL\text{-}17^+$ lamina propria mononuclear cells in the *Hp*-

infected gastric mucosa were $CD3^+$ (Caruso et al., 2008), but there may well be a higher proportion of $CD3^-$ IL-17-producers in the gastric epithelium.

PMA/ionomycin was used to stimulate the gastric biopsy cells prior to flow cytometry analysis, as the number of $IL\text{-}17^+$ events from the numbers of gastric cells available using antigenic stimulation was too small for meaningful analysis. This approach has been used by a number of other groups investigating gastric T cell responses to *Hp* (Bamford et al., 1998, Sommer et al., 1998, Caruso et al., 2008).

7.1.3.2 Peripheral blood Th17 response to Hp

In the peripheral blood there are many more T cells present which are likely to have a wider range of specificities than the gastric T cells. Use of the activation markers CD69 and CD154 was initially investigated to see if they could help to identify antigen-specific $CD4^+$ T cell poulations, but the vast majority of $CD4^+$cytokine$^+$ events were also activation marker positive so this approach did not seem to add much. There was a greater Th17 response to *Hp* antigen in PBMCs from *Hp*-infected patients than PBMCs from uninfected patients. When the PBMCs were cultured with medium only, or tetanus toxoid (control antigen), no difference in Th17 responses was seen between the *Hp*-infected and uninfected groups. This is good evidence that the response is indeed *Hp*-specific. The *Hp*-specific Th17 responses were of similar magnitude to Th17 responses to *C. albicans,* which is known to provoke a Th17 response and causes chronic infection in patients with defects in Th17 pathways. A $CD4^+IFN\gamma^+$ Th1 response to *Hp* was also detected in the peripheral blood, consistent with published findings (Kenefeck, 2008, Quiding-Jarbrink et al., 2001b, Ren et al., 2000). The magnitude of the *Hp*-specific Th1 response was very similar to that of the *Hp*-specific Th17 response. The proportion of $CD4^+IL\text{-}17^+$ co-staining for IFNγ was also investigated. The median $\%CD4^+IL\text{-}17^+$ also staining for was 33% in the *Hp*+ PBMCs, suggesting that this could be a significant population, though it is only a minority of Th17 cells in the peripheral blood of most *Hp*-infected patients.

7.1.3.3 Peripheral blood Treg responses to Hp and the balance of pro-inflammatory and anti-inflammatory responses

Peripheral blood $CD4^+CD25^{high}$ Tregs were increased in *Hp* infection but $CD4^+CD25^{high}FOXP3^+$ and $CD4^+CD25^{high}CD127^{low}$ Tregs were not. This is consistent with published findings (Satoh et al.). There were approximately 1000 times more $CD4^+CD25^{high}$ Tregs than *Hp*-stimulated Th1 or Th17 cells. However, only approximately 1 in 300 $CD4^+CD25^{high}$ Tregs produce IL-10 in response to *Hp* stimulation (Kenefeck, 2008). The data from this study does not allow direct comparison of cytokine secreting Treg with Th1 and Th17 levels, but the available evidence suggests a dominant Treg response. This is consistent with the lack of systemic inflammatory symptoms in *Hp*-infected patients and the possible protective effects of the *Hp*-induced Treg response against allergy and autoimmunity.

Relative levels of pro-inflammatory Th17 and Th1 cells and anti-inflammatory Tregs in the gastric mucosa are likely to influence *Hp* colonization density levels and disease. Gastric Th1 and Treg levels were not measured in this study, therefore direct comparisons at the cellular level cannot be made. However, as Th17 cells can co-produce pro-inflammatory cytokines such as IFNγ or anti-inflammatory IL-10, and these key cytokines can also be produced by cells other than $CD4^+$ T cells, gastric mucosal levels of the relevant cytokines may be more informative than Th cell frequencies. IL-17 levels were significantly higher than IL-10 levels in this study, but levels of anti-inflammatory TGF-β and other pro-inflammatory cytokines may also be critical. Cytokines may differ in their potencies, so comparing absolute concentrations may not give an accurate picture of their relative biological importance. Novel findings regarding IL-17 and Th17 cells and their influence on histopathological findings and disease are discussed next.

7.1.4 Influence of IL-17 and Th17 Cells on Histopathology and Disease

7.1.4.1. Association of Th17 markers with inflammation, activity and Hp density scores

High gastric IL-17 levels were associated with high inflammation and activity scores upon grouped analysis by Mann-Whitney U-test, though the association did not quite reach significance when the data were analyzed using the Kruskal-Wallis test (section 5.3.6). This is consistent with the known role of IL-17 in neutrophils and lymphocyte recruitment. The Sydney scoring system used here is category based (Dixon et al., 1996). It is possible that more statistically significant differences might be detected using a fully quantitative histopathology scoring system, which would provide greater statistical power. There were similar, but weaker trends for an association between *RORC2* expression and inflammation and activity scores (section 6.3.5). The group with the highest inflammation and activity scores was small (n=3). This group appeared to have lower *RORC2* expression, perhaps due to the presence of relatively high numbers of other types of immune cells, such as neutrophils, but firm conclusions cannot be drawn from such a small subset of samples.

There was a trend for higher *RORC2* expression levels to be associated with higher *Hp* density scores, but this did not reach statistical significance (Figure 6.12B). IL-17 levels were lower in those with high *Hp* density than those with intermediate scores. The same pattern was seen for IL-8 levels. This could reflect expansion of the infection in the face of a limited IL-17/IL-8 response.

7.1.4.2 Association of Th17 markers with clinical disease

No significant difference was seen in IL-17 levels or *RORC2* expression between *Hp*-infected patients with and without precancerous changes (atrophy and intestinal metaplasia). This argues against a key role for IL-17/Th17 cells in early malignant transformation in the *Hp*-infected stomach which is consistent with previous findings (Mizuno et al., 2005). However, the

numbers of patients with precancerous changes were quite small and the changes were mostly of a low grade, so these findings need to be interpreted with a degree of caution. Th17 cells are known to be present in gastric cancer, and are found in increasing numbers with more advanced stage disease (Zhang et al., 2008a, Iida et al., 2011). The findings presented here suggest that these Th17 cells may be recruited or differentiated in the tumour microenvironment following carcinogenesis.

Interestingly, although IL-17 levels did not correlate with peptic ulcer disease at the mRNA or protein level, there was an association between high *RORC2* expression and peptic ulcer disease (Figure 6.13). The increase in risk of peptic ulceration with high *RORC2* levels was small, and only just statistically significant, so this needs to be interpreted with some caution. A link between IL-17 and peptic ulcer disease cannot completely be excluded. Gastric mucosal IL-17 concentrations vary considerably between individuals and an association with peptic ulcer disease may be detected in larger studies or studies in different populations. However these findings make it unlikely that IL-17 has a very sizeable effect on peptic ulcer disease risk. The association between RORC2 mRNA expression and peptic ulcer disease but not IL-17 and peptic ulceration raises the possibility that a factor produced by Th17 cells other than IL-17, or a combination of factors, could contribute to this pathology. In the EAE autoimmune disease model Th17-derived GM-CSF is important for pathogenesis (Codarri et al., 2011, El-Behi et al., 2011). However, only low levels of GM-CSF were detected in the gastric mucosa, which were not increased during *Hp*-infection.

7.1.4.3 A role for IFNγ-secreting Th17 cells in Hp-related disease?

$CD4^+IL\text{-}17^+IFN\gamma^+$ cells are well described and are pathogenic in the EAE mouse model and human juvenile idiopathic arthritis (Abromson-Leeman et al., 2009, Cosmi et al., 2011). A proportion of *Hp*-specific Th17 cells were found to co-express IFNγ in the peripheral blood. IL-17 and IFNγ co-expression by gastric $CD4^+$ T cells was not investigated in this study but *IL17* and *IFNG* mRNA showed a trend towards correlation (Spearman's $\rho=0.4$, $p=0.051$). $CD4^+$IL-

$17^+IFN\gamma^+$ responses to other infectious agents including *C. albicans* and tuberculosis have been documented (Zielinski et al., 2012, Jurado et al., 2012). Indeed, in a fate mapping study nearly all of the IFNγ response to *C. albicans* was Th17-derived (Hirota et al.). $CD4^+IL\text{-}17^+IFN\gamma^+$ cells co-express the Th17 and Th1 transcription factors RORγt and T-bet and are derived from Th17 cells in the context of IL-1β, IL-12, IL-23 and low TGF- β (Zielinski et al., 2012, Boniface et al., 2010, Abromson-Leeman et al., 2009, Nistala et al., 2010, Ghoreschi et al., 2010, Volpe et al., 2008). In this study high levels of IL-23 were detected in the gastric mucosa of both *Hp*-infected and uninfected patients, which could drive pathogenic Th17 populations following recruitment to the *Hp*-infected stomach. Only low levels of IFNγ, IL-1β and IL-12 were detected in gastric mucosal biopsies using Luminex, with no difference between the *Hp*-infected and uninfected, or *Hp*-infected with peptic ulcer and *Hp*-infected ulcer-free groups. It may not have been possible to detect differences between the groups due to the very low levels detected using this technology. Gastric stromal factors can reduce DC IL-12 production and Th1 responses (Bimczok et al., 2011). On the other hand numerous studies have found increased IFNγ in the *Hp*-infected gastric mucosa using techniques including immunohistochemistry, ELISA, EliSPOT, cytokine bead array and flow cytometry with *Hp* stimulation (Lindholm et al., 1998, Lehmann et al., 2002, Holck et al., 2003, Bontems et al., 2003, Shimizu et al., 2004, Robinson et al., 2008). Pellicano *et al.* found increased IL-12, IFNγ and t-bet by ELISA and western blotting. Studies of *Hp* infection in Mongolian gerbils found high IFNγ levels in those with ulcers (Yamaoka et al., 2005). Taking the evidence as a whole, it still seems likely that IFNγ (derived from $t\text{-}bet^+$Th17 and/or Th1 cells) has a role in ulcer pathogenesis.

TGF-β was not measured in this study, but is known to be increased in the *Hp*-infected gastric mucosa and is produced by Tregs, which are associated with protection against peptic ulcer disease (Robinson et al., 2008). It is possible that the association of high levels of Tregs with low risk of peptic ulceration is

partly due to their production of TGF-β inhibiting Th17 differentiation to an IFNγ-producing phenotype.

7.1.4.4 Relative gastric IL-17 and IFNγ concentrations

The finding that IL-17 concentrations were 3.9-fold higher than IFNγ concentrations in the *Hp+* gastric biopsies was striking. Samples were spiked with known concentrations of IFNγ and IL-12 to confirm that the Luminex assay was able to detect these cytokines satisfactorily. Few published studies have measured IFNγ concentrations directly comparable with the results here. Shimizu *et al.* measured IFNγ in the supernatants of homogenized gastric biopsies from *Hp*-infected children by ELISA and found mean concentrations of approximately 30 pg/mg protein. In the current study mean concentration in the *Hp+* biopsies was 9.1 pg/mg protein; a little lower but of the same order of magnitude. Serelli-Lee *et al.* measured IL-17 and IFNγ in supernatants from lamina propria mononuclear cells cultured with phytohaemagglutinin and IL-2 by ELISA, but used biopsy weight rather than total protein to adjust for biopsy size. In contrast to findings of the current study Serelli-Lee *et al.* reported median IL-17 concentrations 89 pg/ml/g tissue (range 61-278) and IFNγ concentrations 173 pg/ml/g tissue (range 56-399) (Serelli-Lee et al., 2012). The *in vitro* culture conditions could have altered the relative cytokine levels but it is possible that differences in the study populations and characteristics of the infecting *Hp* strains could account for some of the discrepancies as the study by Serelli-Lee *et al.* was based in Singapore.

The relative levels of cytokines might not necessarily reflect their relative biological activity, indeed the potency of a single cytokine for different functions may vary (Mire-Sluis et al., 1998). Bioassays are required to assess cytokine function but are less well standardized than quantitative immunoassays. Both the Luminex and RT-qPCR methods used here assessed cytokine levels in whole gastric biopsies, so it is possible that high localized levels could occur at the site of disease, though no increase in total IL-17 or

IFNγ levels was seen in those with peptic ulcer disease. Mizuno *et al.* cultured gastric biopsies for 48 hours and measured IL-17 levels in the supernatants. In keeping with the results presented here they found no difference in IL-17 levels between the gastric ulcer and non-ulcer patients. However, they found increased IL-17 levels at the ulcer site compared to normal antral tissue (Mizuno et al., 2005).

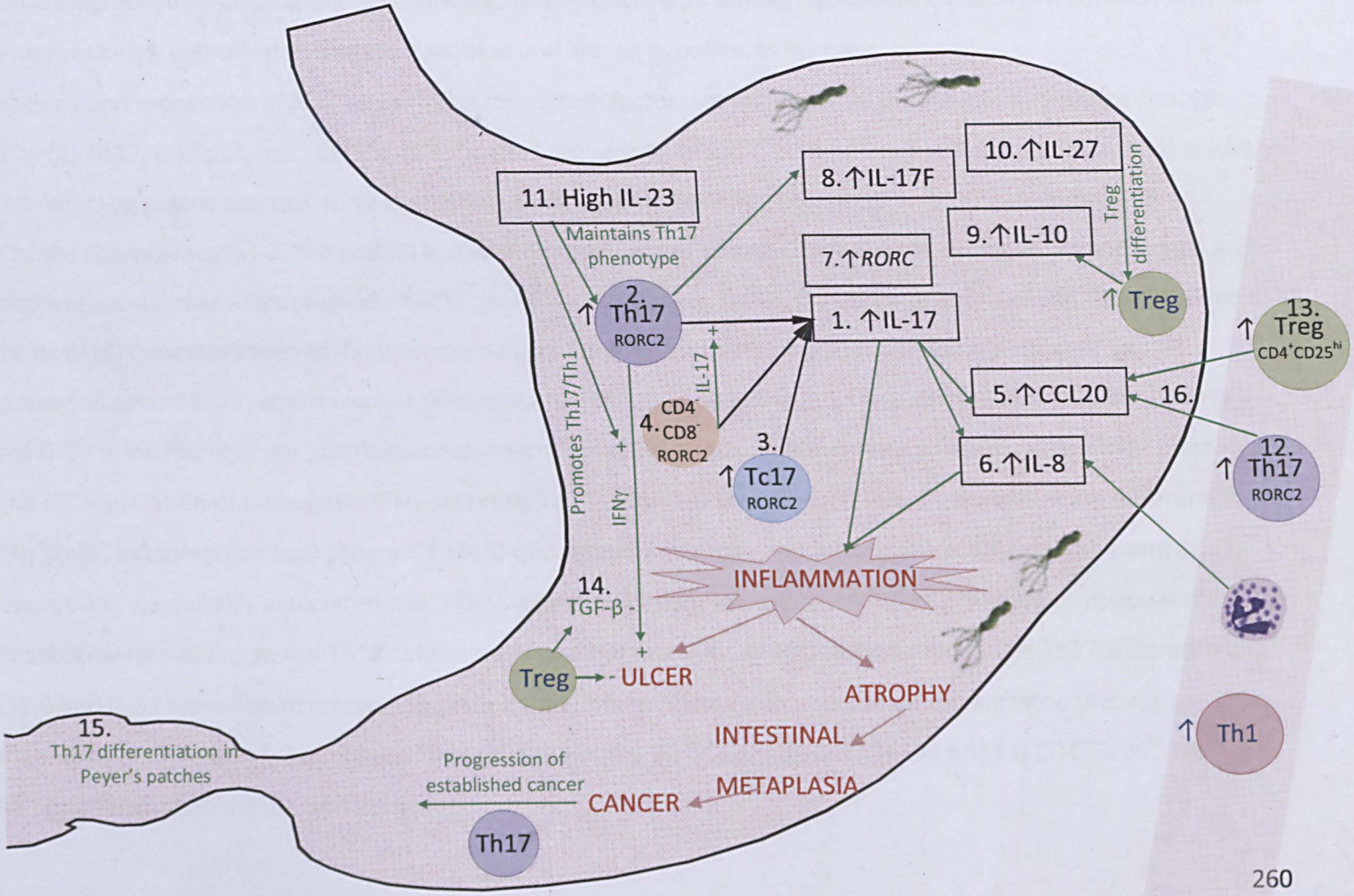

11. High IL-23
Maintains Th17 phenotype
2. Th17 RORC2
8.↑IL-17F
10.↑IL-27
9.↑IL-10
7.↑RORC
1. ↑IL-17
Treg differentiation
Treg
13. Treg CD4+CD25hi
5.↑CCL20
16.
12. Th17 RORC2
6.↑IL-8
Promotes Th17/Th1
IFNγ
IL-17
4. CD4- CD8- RORC2
3. Tc17 RORC2
INFLAMMATION
14. TGF-β
Treg
ULCER
ATROPHY
INTESTINAL METAPLASIA
CANCER
Progression of established cancer
Th17
15. Th17 differentiation in Peyer's patches
Th1

Figure 7.1 Schematic diagram summarizing the main findings of the study with linking hypotheses. Findings from this study are shown in black, with previously published findings in dark blue and linking hypotheses in green.

(1) IL-17 concentrations and expression of (7) The *RORC2* transcription factor, are increased in the *Hp*-infected gastric mucosa. IL-17 is produced by (2) Th17, (3) Tc17, and (4) $CD4^-CD8^-$ T cells. Frequencies of $IL\text{-}17^+$ cells of T cell subsets (2), (3) and (4) are all increased in the *Hp*-infected gastric mucosa. IL-17 is associated with gastric inflammation which it may contribute to by increasing levels of the chemokines (5) CCL20 and (6) IL-8 which are known to attract $CCR6^+$ lymphocytes (Th17s and Tregs) and neutrophils respectively. IL-17 may also upregulate G-CSF and GM-CSF (Gaffen, 2008). (8) Concentrations of the Th17 cytokine IL-17F are also increased. (9) Concentrations of IL-10, which may be Treg-derived, were increased in the *Hp*-infected gastric mucosa. (10) Concentrations of IL-27, which favours differentiation of IL-10-producing Tr1 Tregs were also increased. (11) High concentrations of IL-23 were found in the gastric mucosa, regardless of *Hp* status. This may help to maintain the Th17 phenotype and/or favour the differentiation of pathogenic IFNγ-secreting Th17 cells. (14) Treg-derived TGF-β may inhibit the differentiation of $CD4^+IL\text{-}17^+IFN\gamma^+$ cells. Inflammation may progress to PUD or precancer (atrophy and intestinal metaplasia), then cancer. (7) *RORC2* mRNA expression was weakly associated with PUD. Neither IL-17 concentrations nor *RORC2* mRNA expression were associated with precancerous changes, but Th17 cells may promote progression of established cancer. The Th17-differentiating cytokines IL-β, IL-6 and IL-21 were not increased, suggesting that differentiation may occur at an extra-gastric site, such as (15) Peyer's patches, and/or Th17 cells may be recruited from the periphery via CCL20 (16). (12) Th17 and (13) $CD4^+CD25^{hi}$ Tregs are increased in the peripheral blood during *Hp* infection.

7.2 LIMITATIONS OF THE WORK

7.2.1 Collection of samples from patients

7.2.1.1 Patients, inclusion and exclusion criteria

Samples were collected from adult patients attending the Queen's Medical Centre for upper gastrointestinal endoscopy. All of the patients were referred due to signs or symptoms suggestive of an abnormality in the upper gastrointestinal tract. The commonest indication was dyspepsia, but others included anaemia, acid reflux and weight loss. The patients were therefore not representative of the general population. Patients with a history of taking PPIs in the preceding two weeks, antibiotics in the preceding 4 weeks or regular non-steroidal anti-inflammatory drugs or immunosuppressants were excluded from the study, though it is possible that not all aspirin and non-steroidal anti-inflammatory use was disclosed. PPIs and antibiotics can reduce the probability of detecting *Hp* and could therefore lead to patients being misclassified as *Hp* negative. Non-steroidal anti-inflammatory drugs are the other main cause of peptic ulceration, so patients taking these, with the exception of low dose aspirin for cardiovascular prophylaxis, were excluded so that *Hp*-induced peptic ulcer disease could be studied. The uninfected patient group tested negative for *Hp* by multiple methods but did not necessarily have completely normal upper gastrointestinal tracts, as might be desired in negative controls. It is possible that some of the *Hp*- patients could have had previous *Hp* infection, but *Hp* serology is usually positive following recent infection. *Hp* is usually eradicated using triple or quadruple therapy with a PPI and multiple antibiotic agents, but the possibility that asymptomatic *Hp* infection could have been eradicated by antibiotics given for other infections in some cases cannot be excluded.

7.2.1.2 Histopathological and clinical findings

Clinical samples and endoscopy findings were taken at a single time point for each patient. Patients may have had previous ulcers that had healed. Many patients had stopped PPI treatment only two weeks prior to endoscopy. This

may not have been sufficient time for detectable erosions or ulcers to recur. Numbers of patients with the precancerous changes of atrophy or intestinal metaplasia in the study were low, and when present the changes were of low grade. These changes develop following many decades of chronic *Hp*-induced gastritis and the time point when patients became symptomatic and are referred for investigation may precede this. Serelli-Lee *et al.* included a group of patients with previous *Hp* infection in their study and found a high prevalence of precancerous changes in this group (Serelli-Lee et al., 2012).

Hp-induced pathology may be patchy: ulcers and cancers usually develop at a single site with relatively normal surrounding mucosa. This raises the possibility of sampling errors. The biopsies sent for histopathological assessment would not have been taken from exactly the same site as the biopsies used for cytokine analysis by Luminex or RT-qPCR. This may have reduced the probability of finding correlations between histopathological parameters and the cytokine and *RORC2* expression data. Advanced endoscopy techniques are now available which can visualize areas of premalignant pathology. Future studies could use this technology to target precancerous tissue, to compare biopsies from damaged and undamaged sites and to assess patchiness of immune cell infiltration.

7.2.1.3 Matching of patient characteristics

Due to the sample size it was not possible to strictly match *Hp*-infected and uninfected patients for age and gender, which may affect immune responses (Naylor et al., 2005, Arnold et al., Karanfilov et al., 1999, Hoffmann et al., 2005, Afshan et al.). However, there was no significant difference in the ages or male:female ratio of the *Hp*-infected and uninfected patients included in the study.

Country of birth is another potentially important factor to consider, as infection often occurs early in life and more virulent strains are more common in certain geographical areas. For example East Asians strains frequently have a particularly virulent form of CagA (Atherton and Blaser,

2009). The questionnaire used to collect patient data could be amended to include detailed information on country of birth for future studies.

As discussed in the Introduction, host genetic variations such as polymorphisms in cytokines including IL-17 can influence risk of *Hp*-related disease (El-Omar et al., 2000, Hou et al., 2007, Shibata et al., 2009). However the ethical approval for this study did not include permission for genotyping. An amendment to the current ethical approvals and consent form could be applied for to allow these factors to be investigated and controlled for in future studies.

7.2.1.4 Subgrouping of Hp-infected patients

This is the first study with high enough numbers of *Hp*+ patients to allow Th17 markers to be stratified by histopathological and clinical findings, however some of the patient groups were disappointingly small, reducing the power of the study. The numbers of *Hp*-infected gastric biopsies analyzed by flow cytometry were relatively small, precluding subgrouping by histopathological findings or disease. It is particularly important to consider the size of the groups where there were negative findings. For example, as discussed above, the numbers of patients with precancerous changes were quite low and the precancerous changes tended to be low grade when they were found. This means that the finding that precancerous changes were not associated with IL-17 levels or *RORC2* expression needs to be interpreted with some caution. Targeting premaligant gastric mucosa with advanced endoscopy techniques and/or including a group of patients with previous *Hp* infection are possible strategies that could be employed to increase numbers of biopsies/patients with precancerous findings.

Similarly, though $CD4^+CD25^{high}$ Tregs were increased in the peripheral blood of *Hp*-infected compared to uninfected patients, no differences were found in $CD4^+CD25^{high}CD127^{low}$ or $CD4^+CD25^{high}FOXP3^+$ Tregs. These negative findings should ideally be confirmed on a larger set of samples, though they are consistent with others' findings (Greenaway et al., 2011, Satoh et al.). The

number of samples studied for which virulence factor data was available was also low but could be expanded as additional strains are genotyped.

This study has generated a number of hypotheses based on findings in clinical samples from patients, but was observational nature. Future work should include mechanistic studies where interventions can be made and conclusions can be drawn about causation (see below).

7.2.2 Methodology Employed

7.2.2.1 In vitro DC work

Preparing equal amounts of viable *Hp* of different strains for stimulation of DCs *in vitro* was technically difficult and DC responses showed wide inter-donor variation, as noted by other authors (Kranzer et al., 2004, Andres et al., 2011). As relatively little is known about the phenotype of gastric DCs, responses of two different DC models to *Hp* were compared. Initially MoDCs were studied as they have been widely used in the study of DC responses to *Hp* and are relatively easy to work with. Subsequently MyDCs were used, which better reflected gastric DC responses and *in vivo* findings. The findings in the current study and by Bimczok *et al.* (Bimczok et al., 2010) raise questions about the relevance of the MoDC model which has been widely used in *Hp* research. Ideally DCs extracted from gastric tissue would be used, but large amounts of gastric tissue would be required to obtain sufficient numbers of DCs. The commonest reason for surgical resection of the stomach locally is malignancy. DCs in these patients may not be typical and the vast majority of patients are treated with chemotherapy or immunosuppressants prior to surgery, so suitable samples were not available for this approach. Even with gastric DCs, simple *in vitro* models cannot reflect the complexity of gastric mucosa *in vivo*. In a recent publication Bimczok *et al.* used "stroma-conditioned media" to try to include the influence of stromal factors in their *in vitro* model and found that this reduced DC-stimulated Th1 responses (Bimczok et al., 2011). Other cell types present in the gastric mucosa *in vivo* but not in *in vitro* models may also have important effects on DCs and T cell

differentiation. In addition to DCs B cells and macrophages can also act as antigen presenting cells (Weaver et al., 1988) (see section 7.3.1).

7.2.2.2 RT-qPCR

Real-time quantitative PCR is a sensitive method for evaluating gene expression, and was well suited to this study where the availability of clinical biopsy material from each patient was limited. Biopsies can be treated with RNA later, stored at -80°C, then processed and analyzed in batches. The exponential amplification that occurs during PCR means that a tiny amount of contamination may have a significant effect on the results obtained, so measures were taken to minimize the risk of this happening and controls were put in place to detect contamination if it occurred.

As whole gastric biopsies were used for RT-qPCR analysis, the data obtained does not give any information on which cells expressed the genes studied. The mRNA level of gene expression may not be reflected at the protein level. RT-qPCR data should therefore be validated by protein data. *IL17* RT-qPCR was validated by IL-17 protein data from Luminex, flow cytometry and ELISA in this study. Unfortunately *RORC2* flow cytometry was not successful, so RORC2 protein expression was not measured. New RORC2 antibodies may become available for future studies or other techniques to quantify RORC2 protein such as immunohistochemistry or chip cytometry (see below) could be explored. Th17 cells by flow cytometry and *RORC2* expression were both increased in *Hp*-infected compared to uninfected patients, but insufficient patients had both these variables measured to assess for correlations.

The method of RT-qPCR analysis used was relative to a pooled control derived from uninfected donor material, rather than being fully quantitative. It is expressed relative to a housekeeping gene (*GAPDH* in this case) to normalize for the total amount of cDNA. The Pfaffl method was used for data analysis, which takes account of the efficiencies of the PCR reactions (Pfaffl, 2001). This is in contrast to the ΔΔCT method, which can only be used when the efficiency of the PCR of the target gene approximates that of the

housekeeping gene (Giulietti et al., 2001). Some authorities recommend including at least three biological replicates per sample (Taylor et al., 2010), but the number of gastric biopsies per patient was limited by ethical approvals and other resources were also limited, so a single biopsy from each patient was used for RT-qPCR with three technical replicates. Analysis of multiple biopsies from each patient could have reduced the effect of patchiness of infection and inflammation.

7.2.2.3 Luminex

Luminex is an attractive technique for maximizing the data obtained from small clinical samples. As multiple cytokines can be quantified in the same assay it can also give useful information about relative cytokine levels and cytokine profiles. Sample volume requirements are lower than for western blotting and ELISA. Sensitivity is similar to ELISA but the lower sample volume requirements avoid the need for dilution. Protein microarrays (also known as protein chips) are another technology that allows quantitation of multiple proteins in a small volume sample. Spots of capture antibody are bound to a support surface, such as a glass slide, to which the sample is added. This can then be probed with detection antibody, typically fluorescence-labelled. This approach can be expensive and a suitable scanner is required to analyze the slides. Luminex panels including our cytokines of interest allowed flexibility, were commercially available and affordable and there was a Bio-Plex 200 analyzer locally so Luminex methodology was selected. Some data obtained using a similar approach to investigate immune responses to *Hp* has been published previously (Serelli-Lee et al., 2012).

Luminex technology is rapidly gaining in popularity and is now in widespread use for a number of clinical applications. However, the multiplex nature of this technique means that assays for different combinations of cytokines and for different types of samples need to be carefully validated and optimized. Some authors have suggested that Luminex should only be used to select promising biomarkers to investigate further (Djoba Siawaya et al., 2008). It is good practice to confirm results by more than one technique, and further

studies are needed to investigate the unexpectedly low Th1 cytokine results found by Luminex. *In vivo* gastric IL-12 and IFNγ levels may be low due to suppression by Tregs and other stromal factors (Bimczok et al., 2011). Again, whole gastric biopsies were used so the Luminex data does not give information about the cellular sources of the cytokines measured.

7.2.2.4 Flow cytometry

Flow cytometry is a powerful technique for characterizing single cells and determining the cellular sources of cytokines. Four colour flow cytometry was used in this study, to ensure that there were no problems with compensation and we could be confident about the data obtained. Extracellular staining, intracellular staining and analysis were often done on separate days, so there would have been a risk of some of the less stable tandem dyes deconjugating prior to analysis. However, use of increasing numbers of colours is becoming more widespread with advances in flow cytometers and availability of fluorochromes with narrow emission spectra. $CD4^+$ T cells in the peripheral blood were co-stained for IL-17 and IFNγ, but as more is now known about the range of phenotypes of Th17 cells, it would be interesting to co-stain gastric Th17 for other cytokines and transcription factors (see future work).

Attempts were made to investigate RORC2 by flow cytometry but limited reagents were available and problems with non-specific staining could not be overcome despite attempts at optimization with lower concentrations of RORC2 antibody and FcR blocking. As this is a relatively new target it is likely that improved reagents will become available, or alternatively RORC2 protein could be studied using other techniques, as discussed above.

Short-term stimulation is used prior to flow cytometry analysis for cytokines. Antigenic stimulation may be more patho-physiological and was used for peripheral blood cells, but PMA/ionomycin stimulation was used for gastric cells in order to obtain sufficient numbers of the events of interest. There is a risk that *in vitro* stimulation could affect cytokine expression, but short-term stimulation should help keep this to a minimum. This can be borne in mind

when interpreting the flow cytometry results in conjunction with results of the Luminex assays, which were performed on snap frozen gastric biopsies and therefore should give a good reflection of *in vivo* cytokine levels. Despite using six gastric biopsies for flow cytometry there were usually only sufficient cells to set up a single stimulated tube and an unstimulated control tube. Ideally larger amounts of gastric tissue and hence larger numbers of gastric cells would be studied but the number of gastric biopsies that can be taken for research is limited by the ethical permission granted. Single biopsies were used for RT-qPCR and Luminex which yielded important data that helped to validate the flow cytometry data.

It is possible that short-term stimulation could skew towards cytokine production by innate immune cells, which are known to produce cytokine rapidly in response to infection *in vivo. Hp* present in biopsies from infected patients could act as an additional stimulus. Cells such as γδ T cells can secrete IL-17 in response to stimulation of pattern recognition receptions such as TLRs, without the need for TCR stimulation (Martin et al., 2009, Sutton et al., 2009, Romi et al., 2011).

The relative amounts of IL-17 produced by its various cellular sources were not investigated. Median fluorescence intensities could give an indication of this, or the different T cell subsets could be isolated and the supernatants from short-term culture assayed for IL-17.

Immunohistochemistry could have been used to study cell surface markers and transcription factors and can also be used for co-localization of cytokines. Advantages of immunohistochemistry are that it is performed on intact sections of tissue, so cells can be localized. There would also be the potential for further analysis of historical samples, whereas flow cytometry is performed on cells isolated from fresh tissue. However, as detection of IL-17 secreting cells was one of the main aims of this study flow cytometry was used.

7.3 FUTURE WORK

7.3.1 Antigen presenting cells in Hp infection

Like gastric DCs, MyDCs produced undetectable IL-12p70 in response to *Hp* stimulation (Bimczok et al., 2010), in contrast to MoDCs which produced high concentrations of IL-12p70. The MoDC model has been widely used in published *Hp* research but the findings here indicate that different types of DC respond differently to *Hp*. There is no published data on IL-23 gastric DC responses to *Hp*. When planning future studies it needs to be borne in mind that models using DCs derived from circulating blood may not reflect gastric DC responses.

The differences in cytokine secretion by MoDCs and MyDCs upon stimulation with wild-type *Hp* strains or *dupA* null mutants were surprisingly small compared to the differences previously observed with peripheral blood monocytes (Hussein et al., 2010). However monocytes are not present in gastric tissue. Macrophages are present in gastric tissue and can act as antigen presenting cells (Fehlings et al., 2012, Quiding-Jarbrink et al., 2010). Like gastric DCs and MyDCs, they produce little IL-12p70 upon *Hp* stimulation (Fehlings et al., 2012). They may have a more similar TLR profile to monocytes than the MoDCs and MyDCs studied here. B cells are also present within the *Hp*-infected gastric mucosa and can act as antigen presenting cells. Work in the *H. felis* model suggests that B cells may have a regulatory role as B cells activated by *Hp* TLR2 induced IL-10-secreting $CD4^{+}CD25^{+}$ Treg (Tr1) cells (Sayi et al., 2011). IL-17 signalling may regulate B cell recruitment, as IL-17RA knockout mice had increased gastric B cells (Algood et al., 2009). Interestingly Th17 cells were recently found to be crucial for B cell switching to IgA production in Peyer's patches in a fate mapping study (Hirota et al., 2013).

An interesting next step would be to look at the effect of *Hp* with and without active *dupA* on monocyte-derived macrophages. MoDCs could be included in the experiment for comparison. Ideally stroma-conditioned media and normal media would both be used to try to assess the effect of the gastric

environment. It might be possible to set up a collaboration with the group that has access to large samples of surgically resected gastric mucosa and has published studies on gastric DCs and stroma-conditioned media (Bimczok et al., 2010, Bimczok et al., 2011). Large samples of human gastric tissue are not available locally at present other than from cancer patients, but would be very valuable for this research if opportunities arose to obtain them in the future.

Some *Hp* strains with intact *cag*PAI that can colonize mice such as B128 and PMSS1 are now available, but all the strains currently used for colonizing mice have disrupted *dupA* genes, making it difficult to investigate *dupA* effects in a mouse model.

The role of MIF in Th1 differentiation in *Hp* also requires further investigation. MIF has been shown to be increased during *Hp* infection and a role for it in carcinogenesis has also been postulated (Wong et al., 2009, Xia et al., 2004, He et al., 2006). It has been suggested that it could be the pro-Th1 factor in the gastric mucosa in the absence of significant IL-12p70 levels (Bimczok et al., 2010). However, Fehlings *et al.* reported that MIF secreted by DCs was reduced upon *Hp* stimulation (Fehlings et al., 2012). Immunohistochemistry could be used to localize the MIF. We did not measure gastric MIF levels with our Luminex analysis, but this could be included in future studies. MIF could be measured in addition to IL-12 family cytokines in the supernatants of *Hp*-stimulated antigen presenting cells.

7.3.2 Gastric cytokine milieu

The finding of low IL-12 and IFNγ in the study was striking. Both Luminex and RT-qPCR studies showed that significantly less IFNγ than IL-17 is expressed in the gastric mucosa, and Tregs (known to be increased in the *Hp*-infected gastric mucosa) and other stromal factors are known to suppress Th1 responses (Bimczok et al., 2011). However, as discussed above, numerous previous studies have found increased IFNγ in *Hp* infection and it has been linked to protection, inflammation, peptic ulcer disease and preneoplastic

changes in animal models (Hitzler et al., 2012b, Sawai et al., 1999, Smythies et al., 2000, Akhiani et al., 2002, Obonyo et al., 2002, Cui et al., 2003, Sayi et al., 2009, Shi et al., 2005), though other studies suggest that it is not essential for protection (Sawai et al., 1999, Flach et al., Garhart et al., 2003). A next step might be to assay IL-17 and IFNγ levels by ELISA in samples similar to those used for the Luminex assays, if the sample volume for the ELISAs does not require so much dilution that the cytokine concentrations drop below detectable levels.

Some cytokines not measured here could also have important roles in the gastric mucosa. TGF-β is important for both Treg and Th17 differentiation and can suppress Th1 differentiation. It has been shown to be increased in *Hp* infection using immunohistochemistry and RT-qPCR and it is produced by gastric epithelial cells in response to *Hp* (Li and Li, 2006, Lindholm et al., 1998, Robinson et al., 2008, Harris et al., 2008, Kandulski et al., 2008, Beswick et al., 2007). TGF-β is secreted and bound to the cell surface in an inactive form. For ELISA and Luminex assays samples can be treated with acid prior to TGF-β analysis to free the surface bound inactive form, but this may not reflect levels of biologically active TGF-β. Acid-activation also precludes multiplex analysis. IL-18, traditionally thought of as a Th1 cytokine, is known to be upregulated in *Hp* infection and can be produced by gastric epithelial cells, and mononuclear cells including DCs (Tomita et al., 2001, Shimada et al., 2008, Yamauchi et al., 2008, Oertli et al., 2012). More recently DC-derived IL-18 has been shown to drive Treg differentiation in a mouse model (Oertli et al., 2012). As discussed above, MIF may be important for Th1 differentiation.

7.3.3 Further characterization of IL-17 producing cells in the Hp-infected gastric mucosa

It is now becoming clear that Th17 cells are not a single uniform population. There is a spectrum from pro-inflammatory to regulatory subtypes, with different cytokine profiles (Peters et al., 2011). To further elucidate the role of Th17 cells in the *Hp*-infected gastric mucosa it would be helpful to check for T-

bet and FOXP3 co-expression with RORC2 and to see which cytokines in addition to IL-17 these cells are secreting. Cytokines of interest that Th17 cells can also produce include IL-17F, IL-21, IL-22, IFNγ, GM-CSF, IL-26 and IL-10 (Volpe et al., 2008, Manel et al., 2008, Wilson et al., 2007, Langrish et al., 2005). The cytokine profile of gastric Th17 cells could be characterized further using flow cytometry with more colours to allow more cytokines (and their co-expression) to be studied in a single sample. Chip cytometry is another approach to be explored. Immunohistochemistry could be used to co-localize cytokines with cell surface markers and transcription factors, as discussed above in section 7.2.2.4.

Alternatively gastric Th17 populations could be isolated. Larger amounts of gastric tissue would be required for this technique. This could be done using capture technology where an antibody complex that can bind cell surface CD45 and secreted IL-17 is used. The surface bound IL-17 can then be labeled for sorting by flow cytometry or magnetic bead enrichment (Streeck et al., 2008). Commercial kits using this technology are now available for human Th17 cells (IL-17 Secretion Assay – Cell Enrichment and Detection Kit (PE), human, Miltenyi Biotech). Brucklacher-Waldert *et al.* found that surface IL-17 is expressed at low levels on stimulated Th17 cells and could be used to identify viable IL-17-secreting cells without the need for a capture antibody (Brucklacher-Waldert et al., 2009a). This could potentially be applied to $CD8^+$, γδ T cell and other IL-17-secreting populations. RT-qPCR could be performed on mRNA extracted from the IL-17 secreting populations, and/or supernatants from short-term culture could be analyzed for the cytokines of interest. Amounts of IL-17 and other cytokines secreted by the different populations could be compared.

Innate IL-17-secreting populations share many characteristics with Th17 cells but have been under-appreciated until recently. This study suggests that these populations are a significant source of gastric IL-17. It will be important to positively identify and further characterize the $CD4^-CD8^-IL\text{-}17^+$ population(s). It would be relatively straightforward to analyze $IL\text{-}17^+$ γδ T cell

frequencies by flow cytometry and NK cell markers, such as CD56 could also be included.

7.3.4 Relationship between gastric Th17 cells and other CD4+ T cells in the Hp-infected gastric mucosa

Another important area for investigation is the relationship between Th17 cells and other $CD4^+$ T cells present in the *Hp*-infected gastric mucosa. Understanding the plasticity of these cells and the influences they have on each other is key to understanding *Hp*-induced pathology and being able to develop appropriate prognostic tests and therapeutic interventions.

The Th17 and Treg lineages are closely related. They both depend on TGF-β for differentiation (Bettelli et al., 2006). A number of factors can influence the balance of Treg/Th17 differentiation, including the availability of AhR ligands (Quintana et al., 2008, Veldhoen et al., 2009). Retinoic acid, IL-35 and IL-27 favour Treg differentiation (Mucida et al., 2007, Niedbala et al., 2007, Pot et al., 2011). The balance is shifted in favour of Th17 cells in the presence of pro-inflammatory cytokines such as IL-6, low concentration TGF-β and pathogen PRRs (Torchinsky et al., 2009, Nyirenda et al., 2011, Bettelli et al., 2006, Veldhoen et al., 2006, Yang et al., 2008b).

Tregs may assist Th17 development by acting as a source of TGF-β and an IL-2 sink (Veldhoen et al., 2006, Chen et al., 2011), but FOXP3 can inhibit Th17 differentiation by antagonizing RORγt (Zhou et al., 2008a, Yang et al., 2008b). Tregs can convert into IL-17-producing cells in pro-inflammatory environments and $FOXP3^+IL\text{-}17^+$ cells, as well as IL-10-producing Th17 cells have been described (Zielinski et al., 2012, Voo et al., 2009, Ghoreschi et al., 2010). Kao *et al.* studied Th17/Treg balance in mouse *Hp* infection and found that *Hp*-stimulated DCs led to a lower Th17:Treg ratio than DCs stimulated with *E. coli* and *A. lwoffi* (Kao et al., 2010). Neutralizing IL-10 and TGF-β or depleting Tregs with an anti-CD25 antibody increased Th17 responses, and the enhanced Th17 responses were associated with reduced *Hp* colonization (Kao et al., 2010).

Th17 cells are also closely related to the Th1 phenotype. Early studies establishing Th17 cells as a separate lineage found that IFNγ suppressed Th17 differentiation (Harrington et al., 2005). Pathogenic $CD4^+IL\text{-}17^+IFN\gamma^+$ cells expressing both RORγt and T-bet are well recognized and seem to be derived from Th17 cells (Annunziato et al., 2007, Zielinski et al., 2012, Shi et al., 2008, Lee et al., 2009, Bending et al., 2009, Hirota et al., Boniface et al., 2010, Cosmi et al., 2011). T-bet can inhibit the Th17 phenotype by suppressing runx1-mediated RORγt activation (Lazarevic et al., 2011). Th17-derived $CD4^+IL\text{-}17^+IFN\gamma^+$ cells can lose their IL-17-secreting capacity and differentiate into $CD4^+T\text{-}bet^+IFN\gamma^+$ cells, which retain some Th17-like characteristics such as CD161 expression and the ability to upregulate defensin genes (Nistala et al., 2010, Boniface et al., 2010). Conversely Th1 cells do not appear to be able to convert to a Th17 phenotype (Shi et al., 2008). This raises questions as to whether $CD4^+T\text{-}bet^+IFN\gamma^+$ cells identified in *Hp* infection are genuine Th1 cells, or a Th17-derived population. Shi *et al.* found that IL-17 rose before IFNγ in murine *Hp* infection, and IL-17 knockout mice had reduced Th1 responses (Shi et al., 2010), which could fit with some "Th1"/IFNγ responses being Th17-derived.

Th17 cells isolated from *Hp*-infected gastric mucosa could be cultured in gastric stroma-conditioned media and normal media to see if the gastric stromal factors influenced the numbers or phenotype of gastric Th17 cells. A similar experiment could be done with Tregs to see if there was any conversion of Tregs to Th17s when cultured with gastric stromal factors. Different ratios of Tregs and Th17 cells could also be co-cultured. CD161 may be a useful additional marker for further studies of Th1 cells.

Animal studies could provide further information about the influence of different Th17 populations on risk of disease.

7.3.5 Bioassays and in vivo models

A major limitation of this study is its observational nature. No interventions were made therefore only associations could be found. *In vitro*

models/bioassays and animal models may help to elucidate the causes of *Hp*-induced pathology. Gastric epithelial cell lines are widely used *in vitro* but are derived from gastric cancers rather than normal gastric epithelium. Mouse studies of *Hp* have been restricted by a lack of pathogenic *Hp* strains that successfully colonize the murine stomach. The related *H. felis* has been used in a number of studies (Pritchard and Przemeck, 2004, Sayi et al., 2009, Roth et al., 1999, Hitzler et al., 2012a). Mongolian gerbils have also been studied as they can be colonized by *Hp* and develop *Hp*-induced pathology, but the available reagents for these animals are limited. Recently a more virulent mouse-colonizing strain of *Hp* named PMSS1 has become available, which is *cagA*+ and is able to transfer the CagA protein into host epithelial cells (Arnold et al., 2011b, Hitzler et al., 2012a). This could be an important tool for investigating *Hp*-related patho-physiology. Hitzler *et al.* studied the development of preneoplastic pathology in response to infection with this strain in $\alpha\beta$ $TCR^{-/-}$, $p19^{-/-}$ (lacking IL-23) and $p35^{-/-}$ (lacking IL-12) mice. The $\alpha\beta$ TCR was required for control of *Hp* infection and development of preneoplastic pathology but neither p19 nor p35 seemed to affect *Hp* colonization densities (Hitzler et al., 2012a). The p19 knockout mice had less gastritis and preneoplastic pathology (Hitzler et al., 2012a). It would be informative to characterize IL-17-secreting cells and other $CD4^+$ cells in the stomachs of wild-type mice infected with the PMSS1 strain. Possible pathogenic or anti-inflammatory populations could then be adoptively transferred to see if this modulated disease.

7.4 SUMMARY OF MAIN FINDINGS AND POSSIBLE CLINICAL IMPLICATIONS

- IL-17 concentrations were significantly higher than IFNγ, IL-4 or IL-10 concentrations in the *Hp*-infected stomach.
- $CD4^+IL\text{-}17^+$ (Th17) cells were the main source of IL-17 in the *Hp*-infected gastric mucosa but $CD8^+IL\text{-}17^+$ (Tc17) and $CD3^+CD4^-CD8^-IL$-

17^+ cell frequencies were also increased. Innate T cell-derived IL-17 could boost IL-17 production by conventional T cells.

- Gastric CCL20 levels were high and markedly increased in *Hp* infection, but IL-1β, IL-6 and IL-21 levels were relatively low and not increased in *Hp* infection. This suggests that *Hp*-specific Th17 cells differentiate at an extra-gastric site (perhaps Peyer's patches) and Th17 cells are recruited to the stomach by CCL20 due to their expression of CCR6.
- High IL-23 levels in the gastric mucosa (though not increased in *Hp* infection) could help to maintain/expand Th17 populations and may promote their development into more inflammatory/pathogenic phenotypes e.g. with IFNγ coexpression.
- High *RORC2* expression was associated with increased risk of peptic ulcer disease, though IL-17 levels were not, suggesting that other Th17 factors (perhaps IFNγ) are involved in peptic ulceration.
- Neither *RORC2* expression nor IL-17 levels were correlated with risk of atrophy or intestinal metaplasia, suggesting that Th17 cells are not involved in carcinogenesis, although they may promote progression of established tumours.
- *Hp*-specific Th17 cells were also increased in the blood of *Hp*-infected patients. A proportion of the *Hp*-specific Th17 cells in the blood also expressed IFNγ.
- Different types of DC have different cytokine responses to *Hp* and it is not clear how well the MoDC or MyDC models reflect gastric DCs.
- Mutation of the dupA virulence factor reduced cytokine secretion by *Hp*-stimulated DCs, though the effect was less marked than that reported for *Hp*-stimulated monocytes.

This study has added substantially to the knowledge of IL-17/Th17 cells in human *Hp* infection. A range of techniques have been used to characterize IL-17/Th17 responses to *Hp* and compare their levels relative to Th1/IFNγ

responses. A strong IL-17 response is a key part of the human immune response to this common pathogen, but may not be directly linked to disease. Data from murine models appears conflicting with reduced *Hp* colonization in IL-17 knockout mice, but increased colonization in IL-17RA knockout mice (Shi et al., 2010, Shiomi et al., 2008, DeLyria et al., 2011, Algood et al., 2009). It is now recognized that Th17 cells are heterogeneous, with some more regulatory Th17s co-expressing IL-10, and other pro-inflammatory Th17s expression IFNγ and/or GM-CSF. This may account for some of the confusion about the role of Th17 cells in *Hp* infection. $CD8^+$ T cells and $CD4^-CD8^-$ T cells are also important sources of IL-17, but little is known about these populations in the context of *Hp* infection.

Further experiments including animal studies are needed to test the hypotheses generated by this study before IL-17/Th17 modulating therapies could be used in the management of patients with *Hp.* In symptomatic patients the first line treatment is usually to eradicate *Hp.* However antibiotic resistance is increasing and a persistent Th17 response may continue, even after successful eradication (Serelli-Lee et al., 2012, Malfertheiner et al., 2012). IL-17/Th17 modulating treatments may become a reality for a high risk group of *Hp*-infected patients in the future.

8. REFERENCES

ABRAHAM, S. N. & ST. JOHN, A. L. (2010) Mast cell-orchestrated immunity to pathogens. *Nat Rev Immunol,* 10, 440-452.

ABROMSON-LEEMAN, S., BRONSON, R. T. & DORF, M. E. (2009) Encephalitogenic T cells that stably express both T-bet and RORgt consistently produce IFNg but have a spectrum of IL-17 profiles. *Journal of Neuroimmunology,* 215, 10-24.

ACOSTA-RODRIGUEZ, E. V., NAPOLITANI, G., LANZAVECCHIA, A. & SALLUSTO, F. (2007a) Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17–producing human T helper cells. *Nature Immunology,* 8, 942-9.

ACOSTA-RODRIGUEZ, E. V., RIVINO, L., GEGINAT, J., JARROSAY, D., GATTORNO, M., LANZAVECCHIA, A., SALLUSTO, F. & NAPOLITANI, G. (2007b) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nature Immunology,* 8, 639-46.

AFSHAN, G., AFZAL, N. & QURESHI, S. CD4+CD25(hi) regulatory T cells in healthy males and females mediate gender difference in the prevalence of autoimmune diseases. *Clinical Laboratory,* 58, 567-71.

AGENCY, H. P. (2011) Identification of Helicobacter species. UK standards for microbiology investigations.

AGGARWAL, S., GHILARDI, N., XIE, M.-H., DE SAUVAGE, F. J. & GURNEY, A. L. (2003) Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *Journal of Biological Chemistry,* 278, 1910-4.

AGNIHOTRI, N., BHASIN, D., VOHRA, H., RAY, P., SINGH, K. & GANGULY, N. (1998) Characterization of lymphocytic subsets and cytokine production in gastric biopsy samples from Helicobacter pylori patients. *Scand J Gastroenterol,* 33, 704-9.

AKHIANI, A. A., PAPPO, J., KABOK, Z., SCHON, K., GAO, W., FRANZEN, L. E. & LYCKE, N. (2002) Protection against Helicobacter pylori infection following immunization is IL-12-dependent and mediated by Th1 cells. *Journal of Immunology,* 169, 6977-84.

AKIMOVA, T., BEIER, U. H., WANG, L., LEVINE, M. H. & HANCOCK, W. W. (2011) Helios expression is a marker of T cell activation and proliferation. *PLoS ONE [Electronic Resource],* 6, e24226.

ALAKKARI, A., ZULLO, A. & O'CONNOR, H. J. (2011) Helicobacter pylori and nonmalignant diseases. *Helicobacter,* 16 Suppl 1, 33-7.

ALAM, M. S., KURTZ, C. C., ROWLETT, R. M., REUTER, B. K., WIZNEROWICZ, E., DAS, S., LINDEN, J., CROWE, S. E. & ERNST, P. B. (2009) CD73 is expressed by human regulatory T helper cells and suppresses proinflammatory cytokine production and Helicobacter felis-induced gastritis in mice. *Journal of Infectious Diseases,* 199, 494-504.

ALGOOD, H. M. S., ALLEN, S. S., WASHINGTON, M. K., PEEK, R. M., JR., MILLER, G. G. & COVER, T. L. (2009) Regulation of gastric B cell recruitment is dependent on IL-17 receptor A signaling in a model of chronic bacterial infection. *Journal of Immunology,* 183, 5837-46.

ALGOOD, H. M. S., GALLO-ROMERO, J., WILSON, K. T., PEEK, R. M. & COVER, T. L. (2007) Host response to Helicobacter pylori infection before initiation of the adaptive immune response. *FEMS,* 51, 577-86.

AMBERBIR, A., MEDHIN, G., ERKU, W., ALEM, A., SIMMS, R., ROBINSON, K., FOGARTY, A., BRITTON, J., VENN, A. & DAVEY, G. (2011) Effects of Helicobacter pylori, geohelminth infection and selected commensal bacteria on the risk of allergic disease and sensitization in 3-year-old Ethiopian children. *Clinical & Experimental Allergy,* 41, 1422-30.

AMEDEI, A., BERGMAN, M. P., APPELMELK, B. J., AZZURRI, A., BENAGIANO, M., TAMBURINI, C., VAN DER ZEE, R., TELFORD, J. L., VANDENBROUCKE-GRAULS, C. M. J. E., D'ELIOS, M. M. & DEL PRETE, G. (2003) Molecular mimicry between Helicobacter pylori antigens and H+, K+ --adenosine triphosphatase in human gastric autoimmunity. *Journal of Experimental Medicine,* 198, 1147-56.

AMEDEI, A., CAPPON, A., CODOLO, G., CABRELLE, A., POLENGHI, A., BENAGIANO, M., TASCA, E., AZZURRI, A., D'ELIOS, M. M., DEL PRETE, G. & DE BERNARD, M. (2006) The neutrophil-activating protein of Helicobacter pylori promotes Th1 immune responses. *Journal of Clinical Investigation,* 116, 1092-101.

AMIEVA, M. R., VOGELMANN, R., COVACCI, A., TOMPKINS, L. S., NELSON, W. J. & FALKOW, S. (2003) Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. *Science,* 300, 1430-4.

ANDERSON, K. M., CZINN, S. J., REDLINE, R. W. & BLANCHARD, T. G. (2006) Induction of CTLA-4-Mediated Anergy Contributes to Persistent Colonization in the Murine Model of Gastric *Helicobacter pylori* Infection. *Journal of Immunology,* 176, 5306-13.

ANDRES, S., SCHMIDT, H.-M. A., MITCHELL, H., RHEN, M., MAEURER, M. & ENGSTRAND, L. (2011) Helicobacter pylori defines local immune response through interaction with dendritic cells. *FEMS Immunology & Medical Microbiology,* 61, 168-78.

ANNUNZIATO, F., COSMI, L., SANTARLASCI, V., MAGGI, L., LIOTTA, F., MAZZINGHI, B., PARENTE, E., FILÌ, L., FERRI, S., FROSALI, F., GIUDICI, F., ROMAGNANI, P., PAOLA PARRONCHI, TONELLI, F., MAGGI, E. & ROMAGNANI, S. (2007) Phenotypic and functional features of human Th17 cells. *The Journal of Experimental Medicine,* 204, 1849-61.

APETOH, L., QUINTANA, F. J., POT, C., JOLLER, N., XIAO, S., KUMAR, D., BURNS, E. J., SHERR, D. H., WEINER, H. L. & KUCHROO, V. K. (2010) The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Journal,* 11, 854-861.

APPELMELK, B. J., VAN DIE, I., VAN VLIET, S. J., VANDENBROUCKE-GRAULS, C. M. J. E., GEIJTENBEEK, T. B. H. & VAN KOOYK, Y. (2003) Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. *Journal of Immunology,* 170, 1635-9.

ARGENT, R. H., HALE, J. L., EL-OMAR, E. M. & ATHERTON, J. C. (2008) Differences in Helicobacter pylori CagA tyrosine phosphorylation motif patterns between western and East Asian strains, and influences on interleukin-8 secretion. *Journal of Medical Microbiology,* 57, 1062-1067.

ARGENT, R. H., KIDD, M., OWEN, R. J., THOMAS, R. J., LIMB, M. C. & ATHERTON, J. C. (2004) Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of Helicobacter pylori. *Gastroenterology,* 127, 514-523.

ARNOLD, C. R., WOLF, J., BRUNNER, S., HERNDLER-BRANDSTETTER, D. & GRUBECK-LOEBENSTEIN, B. Gain and loss of T cell subsets in old age--age-related reshaping of the T cell repertoire. *Journal of Clinical Immunology,* 31, 137-46.

ARNOLD, D. M., BERNOTAS, A., NAZI, I., STASI, R., KUWANA, M., LIU, Y., KELTON, J. G. & CROWTHER, M. A. (2009) Platelet count response to H. pylori treatment in patients with immune thrombocytopenic purpura with and without H. pylori infection: a systematic review. *Haematologica,* 94, 850-6.

ARNOLD, I. C., DEHZAD, N., REUTER, S., MARTIN, H., BECHER, B., TAUBE, C. & MULLER, A. (2011a) Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *Journal of Clinical Investigation,* 121, 3088-93.

ARNOLD, I. C., LEE, J. Y., AMIEVA, M. R., ROERS, A., FLAVELL, R. A., SPARWASSER, T. & MULLER, A. (2011b) Tolerance Rather Than Immunity Protects From Helicobacter pylori-Induced Gastric Preneoplasia. *Gastroenterology,* 140, 199-209.e8.

ARRUVITO, L., SANZ, M., BANHAM, A. H. & FAINBOIM, L. (2007) Expansion of CD4+CD25+and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction. *Journal of Immunology,* 178, 2572-8.

ATARASHI, K., NISHIMURA, J., SHIMA, T., UMESAKI, Y., YAMAMOTO, M., ONOUE, M., YAGITA, H., ISHII, N., EVANS, R., HONDA, K. & TAKEDA, K. (2008) ATP drives lamina propria TH17 cell differentiation. *Nature,* 455, 808-812.

ATHERTON, J. C. (2006) The pathogenesis of *Helicobacter pylori*-induced gastro-duodenal diseases. *Annual Review of Pathological Mechanisms of Disease,* 1, 63-96.

ATHERTON, J. C. & BLASER, M. J. (2009) Coadaptation of Helicobacter pylori and humans: ancient history, modern implications. *The Journal of Clinical Investigation,* 119, 2475-2487.

ATHERTON, J. C., CAO, P., PEEK, R. M., TUMMURU, M. K. R., BLASER, M. J. & COVER, T. L. (1995) Mosaicism in Vacuolating Cytotoxin Alleles of Helicobacter pylori: ASSOCIATION OF SPECIFIC vacA TYPES WITH CYTOTOXIN PRODUCTION AND PEPTIC ULCERATION. *Journal of Biological Chemistry,* 270, 17771-17777.

AUJLA, S. J., CHAN, Y. R., ZHENG, M., FEI, M., ASKEW, D. J., POCIASK, D. A., REINHART, T. A., MCALLISTER, F., EDEAL, J., GAUS, K., HUSAIN, S., KREINDLER, J. L., DUBIN, P. J., PILEWSKI, J. M., MYERBURG, M. M., MASON, C. A., IWAKURA, Y. & KOLLS, J. K. (2008) IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nature Medicine,* 14, 275-81.

AVILES-JIMENEZ, F., LETLEY, D. P., GONZALEZ-VALENCIA, G., SALAMA, N., TORRES, J. & ATHERTON, J. C. (2004) Evolution of the Helicobacter pylori Vacuolating Cytotoxin in a Human Stomach. *Journal of Bacteriology,* 186, 5182-5185.

AWASTHI, A., CARRIER, Y., PERON, J. P. S., BETTELLI, E., KAMANAKA, M., FLAVELL, R. A., KUCHROO, V. K., OUKKA, M. & WEINER, H. L. (2007) A dominant function for interleukin 27 in generating interleukin 10–producing anti-inflammatory T cells. *Nature Immunology,* 8, 1380-9.

AZUMA, T., YAMAZAKI, S., YAMAKAWA, A., OHTANI, M., MURAMATSU, A., SUTO, H., ITO, Y., DOJO, M., YAMAZAKI, Y., KURIYAMA, M., KEIDA, Y., HIGASHI, H. & HATAKEYAMA, M. (2004) Association between Diversity in the Src Homology 2 Domain-Containing Tyrosine Phosphatase Binding Site of Helicobacter pylori CagA Protein and Gastric Atrophy and Cancer. *Journal of Infectious Diseases,* 189, 820-827.

BAE, J. M., SHIN, S.-H., KWON, H.-J., PARK, S.-Y., KOOK, M. C., KIM, Y.-W., CHO, N.-Y., KIM, N., KIM, T.-Y., KIM, D. & KANG, G. H. ALU and LINE-1 hypomethylations in multistep gastric carcinogenesis and their prognostic implications. *International Journal of Cancer,* 131, 1323-1331.

BAECHER-ALLAN, C., WOLF, E. & HAFLER, D. A. (2005) Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4+CD25+ T cells. *Clinical Immunology,* 115, 10-18.

BAMBA, N., NAKAJIMA, S., ANDOH, A., BAMBA, M., SUGIHARA, H., BAMBA, T. & HATTORI, T. (2002) Stem Cell Factor Expressed in Human Gastric Mucosa in Relation to Mast Cell Increase in Helicobacter pylori-Infected Gastritis. *Digestive Diseases and Sciences,* 47, 274-282.

BAMFORD, K. B., FAN, X., CROWE, S. E., LEARY, J. F., GOURLEY, W. K., LUTHRA, G. K., BROOKS, E. G., GRAHAM, D. Y., REYES, V. E. & ERNST, P. B. (1998) Lymphocytes in the Human Gastric Mucosa During *Helicobacter pylori* Have a T Helper Cell 1 Phenotype. *Gastroenterology,* 114, 482-492.

BANDYOPADHYAY, A. & RAGHAVAN, S. (2009) Defining the role of integrin alphavbeta6 in cancer. *Current Drug Targets,* 10, 645-52.

BARRETT, J. C., HANSOUL, S., NICOLAE, D. L., CHO, J. H., DUERR, R. H., RIOUX, J. D., BRANT, S. R., SILVERBERG, M. S., TAYLOR, K. D., BARMADA, M. M., BITTON, A., DASSOPOULOS, T., DATTA, L. W., GREEN, T., GRIFFITHS, A. M., KISTNER, E. O., MURTHA, M. T., REGUEIRO, M. D., ROTTER, J. I., SCHUMM, L. P., STEINHART, A. H., TARGAN, S. R., XAVIER, R. J., CONSORTIUM, N. I. G., LIBIOULLE, C., SANDOR, C., LATHROP, M., BELAICHE, J., DEWIT, O., GUT, I., HEATH, S., LAUKENS, D., MNI, M., RUTGEERTS, P., VAN GOSSUM, A., ZELENIKA, D., FRANCHIMONT, D., HUGOT, J.-P., DE VOS, M., VERMEIRE, S., LOUIS, E., BELGIAN-FRENCH, I. B. D. C., WELLCOME TRUST CASE CONTROL, C., CARDON, L. R., ANDERSON, C. A., DRUMMOND, H., NIMMO, E., AHMAD, T., PRESCOTT, N. J., ONNIE, C. M., FISHER, S. A., MARCHINI, J., GHORI, J., BUMPSTEAD, S., GWILLIAM, R., TREMELLING, M., DELOUKAS, P., MANSFIELD, J., JEWELL, D., SATSANGI, J., MATHEW, C. G., PARKES, M., GEORGES, M. & DALY, M. J. (2008) Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nature Genetics,* 40, 955-62.

BATTEN, M., LI, J., YI, S., KLJAVIN, N. M., DANILENKO, D. M., LUCAS, S., LEE, J., DE SAUVAGE, F. J. & GHILARDI, N. (2006) Interleukin 27 limits autoimmune encephalomyelitis by

suppressing the development of interleukin 17-producing T cells. *Nat Immunol,* 7, 929-936.

BAUER, B., PANG, E., HOLLAND, C., KESSLER, M., BARTFELD, S. & MEYER, THOMASÂ F. (2012) The Helicobacter pylori Virulence Effector CagA Abrogates Human Î²-Defensin 3 Expression via Inactivation of EGFR Signaling. *Cell Host & Microbe,* 11, 576-586.

BAUER, B., WEX, T., KUESTER, D., MEYER, T. & MALFERTHEINER, P. (2013) Differential Expression of Human Beta Defensin 2 and 3 in Gastric Mucosa of Helicobacter pylori-Infected Individuals. *Helicobacter,* 18, 6-12.

BAUMGARTH, N. & ROEDERER, M. (2000) A practical approach to multicolor flow cytometry for immunophenotyping. *Journal of Immunological Methods,* 243, 77-97.

BAUQUET, A. T., JIN, H., PATERSON, A. M., MITSDOERFFER, M., HO, I. C., SHARPE, A. H. & KUCHROO, V. K. (2009) The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nature Immunology,* 10, 167-75.

BECHER, B., DURELL, B. G. & NOELLE, R. J. (2002) Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *Journal of Clinical Investigation,* 110, 493-7.

BECKER, C., WIRTZ, S., BLESSING, M., PIRHONEN, J., STRAND, D., BECHTHOLD, O., FRICK, J., GALLE, P. R., AUTENRIETH, I. & NEURATH, M. F. (2003) Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. *Journal of Clinical Investigation,* 112, 693-706.

BEN-NERIAH, Y. & KARIN, M. (2011) Inflammation meets cancer, with NF-kappaB as the matchmaker. *Nature Immunology,* 12, 715-23.

BENDING, D., DE LA PEÑA, H., VELDHOEN, M., PHILLIPS, J. M., UYTTENHOVE, C., STOCKINGER, B. & COOKE, A. (2009) Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *Journal of Clinical Investigation,* 119, 565-72.

BENOIT, M., DESNUES, B. & MEGE, J.-L. (2008) Macrophage polarization in bacterial infections. *Journal of Immunology,* 181, 3733-9.

BERGMAN, M. P., ENGERING, A., SMITS, H. H., VAN VLIET, S. J., VAN BODEGRAVEN, A. A., WIRTH, H.-P., KAPSENBERG, M. L., VANDENBROUCKE-GRAULS, C. M. J. E., VAN KOOYK, Y. & APPELMELK, B. J. (2004) Helicobacter pylori modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. *Journal of Experimental Medicine,* 200, 979-90.

BESWICK, E. J., PINCHUK, I. V., DAS, S., POWELL, D. W. & REYES, V. E. (2007) Expression of the programmed death ligand 1, B7-H1, on gastric epithelial cells after Helicobacter pylori exposure promotes development of CD4+ CD25+ FoxP3+ regulatory T cells. *Infection & Immunity,* 75, 4334-41.

BETTELLI, E., CARRIER, Y., GAO, W., KORN, T., STROM, T. B., OUKKA, M., WEINER, H. L. & KUCHROO, V. K. (2006) Reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T cells. *Nature,* 441, 235-238.

BILLERBECK, E., KANG, Y.-H., WALKER, L., LOCKSTONE, H., GRAFMUELLER, S., FLEMING, V., FLINT, J., WILLBERG, C. B., BENGSCH, B., SEIGEL, B., RAMAMURTHY, N., ZITZMANN, N., BARNES, E. J., THEVANAYAGAM, J., BHAGWANANI, A., LESLIE, A., OO, Y. H., KOLLNBERGER, S., BOWNESS, P., DROGNITZ, O., ADAMS, D. H., BLUM, H. E., THIMME, R. & KLENERMAN, P. (2010) Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. *Proceedings of the National Academy of Sciences of the United States of America,* 107, 3006-11.

BIMCZOK, D., CLEMENTS, R. H., WAITES, K. B., NOVAK, L., ECKHOFF, D. E., MANNON, P. J., SMITH, P. D. & SMYTHIES, L. E. Human primary gastric dendritic cells induce a Th1 response to H. pylori. *Mucosal Immunol.*

BIMCZOK, D., CLEMENTS, R. H., WAITES, K. B., NOVAK, L., ECKHOFF, D. E., MANNON, P. J., SMITH, P. D. & SMYTHIES, L. E. (2010) Human primary gastric dendritic cells induce a Th1 response to H. pylori. *Mucosal Immunol,* 3, 260-269.

BIMCZOK, D., GRAMS, J. M., STAHL, R. D., WAITES, K. B., SMYTHIES, L. E. & SMITH, P. D. (2011) Stromal regulation of human gastric dendritic cells restricts the Th1 response to Helicobacter pylori. *Gastroenterology,* 141, 929-38.

BIRKHOLZ, S., KNIPP, U. & OPFERKUCH, W. (1993) Stimulatory effects of Helicobacter pylori on human peripheral blood mononuclear cells of H. pylori infected patients and healthy blood donors. *Zentralblatt fur Bakteriologie,* 280, 166-76.

BLASER, M. J. & ATHERTON, J. C. (2004) *Helicobacter pylori* persistence: biology and disease. *Journal of Clinical Investigation,* 113, 321-333.

BLASER, M. J., CHEN, Y. & REIBMAN, J. (2008) Does Helicobacter pylori protect against asthma and allergy? *Gut,* 57, 561-7.

BODGER, K., WYATT, J. I. & HEATLEY, R. V. (1997) Gastric mucosal secretion of interleukin-10: relations to histopathology, Helicobacter pylori status, and tumour necrosis factor-alpha secretion. *Gut,* 40, 739-44.

BOGSTEDT, A. K., NAVA, S., WADSTROM, T. & HAMMARSTROM, L. (1996) Helicobacter pylori infections in IgA deficiency: lack of role for the secretory immune system. *Clinical & Experimental Immunology,* 105, 202-4.

BONIFACE, K., BLUMENSCHEIN, W. M., BROVONT-PORTH, K., MCGEACHY, M. J., BASHAM, B., DESAI, B., PIERCE, R., MCCLANAHAN, T. K., SADEKOVA, S. & DE WAAL MALEFYT, R. (2010) Human Th17 Cells Comprise Heterogeneous Subsets Including IFNγ Producing Cells with Distinct Properties from the Th1 Lineage. *The Journal of Immunology,* 185, 679-687.

BONTEMS, P., ROBERT, F., VAN GOSSUM, A., CADRANEL, S. & MASCART, F. (2003) Helicobacter pylori modulation of gastric and duodenal mucosal T cell cytokine secretions in children compared with adults. *Helicobacter,* 8, 216-26.

BONVALET, M., WAMBRE, E., MOUSSU, H., HORIOT, S., KWOK, W. W., LOUISE, A., EBO, D., HOARAU, C., VAN OVERTVELT, L., BARON-BODO, V. & MOINGEON, P. Comparison between major histocompatibility complex class II tetramer staining and surface expression of activation markers for the detection of allergen-specific CD4[SUPERSCRIPT PLUS SIGN] T cells. *Clinical & Experimental Allergy,* 41, 821-9.

BOREN, T., FALK, P., ROTH, K. A., LARSON, G. & NORMARK, S. (1993) Attachment of Helicobacter pylori to human gastric epithelium mediated by blood group antigens. *Science,* 262, 1892-5.

BOUGHAN, P. K., ARGENT, R. H., BODY-MALAPEL, M., PARK, J.-H., EWINGS, K. E., BOWIE, A. G., ONG, S. J., COOK, S. J., SORENSEN, O. E., MANZO, B. A., INOHARA, N., KLEIN, N. J., NUNEZ, G., ATHERTON, J. C. & BAJAJ-ELLIOTT, M. (2006) Nucleotide-binding oligomerization domain-1 and epidermal growth factor receptor: critical regulators of beta-defensins during Helicobacter pylori infection. *Journal of Biological Chemistry,* 281, 11637-48.

BRANDT, K., BULFONE-PAUS, S., FOSTER, D. C. & RUCKERT, R. (2003) Interleukin-21 inhibits dendritic cell activation and maturation. *Blood,* 102, 4090-8.

BRIGHT, J. J., MUSURO, B. F., DU, C. & SRIRAM, S. (1998) Expression of IL-12 in CNS and lymphoid organs of mice with experimental allergic encephalitis. *Journal of Neuroimmunology,* 82, 22-30.

BRUCE, M. G. & MAAROOS, H. I. (2008) Epidemiology of Helicobacter pylori infection. *Helicobacter,* 13 Suppl 1, 1-6.

BRUCKLACHER-WALDERT, V., STEINBACH, K., LIOZNOV, M., KOLSTER, M., HÃSCHER, C. & TOLOSA, E. (2009a) Phenotypical Characterization of Human Th17 Cells Unambiguously Identified by Surface IL-17A Expression. *The Journal of Immunology,* 183, 5494-5501.

BRUCKLACHER-WALDERT, V., STUERNER, K., KOLSTER, M., WOLTHAUSEN, J. & TOLOSA, E. (2009b) Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. *Brain,* 132, 3329-41.

BRUSTLE, A., HEINK, S., HUBER, M., ROSENPLANTER, C., STADELMANN, C., YU, P., ARPAIA, E., MAK, T. W., KAMRADT, T. & LOHOFF, M. (2007) The development of inflammatory TH-17 cells requires interferon-regulatory factor 4. *Nature Immunology,* 8, 958-66.

BUCKLAND, G., AGUDO, A., LUJÃN, L., JAKSZYN, P., BUENO-DE-MESQUITA, H. B., PALLI, D., BOEING, H., CARNEIRO, F. T., KROGH, V., SACERDOTE, C., TUMINO, R., PANICO, S.,

NESI, G., MANJER, J., REGNÃR, S., JOHANSSON, I., STENLING, R., SANCHEZ, M.-J., DORRONSORO, M., BARRICARTE, A., NAVARRO, C., QUIRÃS, J. R., ALLEN, N. E., KEY, T. J., BINGHAM, S., KAAKS, R., OVERVAD, K., JENSEN, M., OLSEN, A., TJÃ¸NNELAND, A., PEETERS, P. H. M., NUMANS, M. E., OCKÃ, M. C., CLAVEL-CHAPELON, F. O., MOROIS, S., BOUTRON-RUAULT, M.-C., TRICHOPOULOU, A., LAGIOU, P., TRICHOPOULOS, D., LUND, E., COUTO, E., BOFFETA, P., JENAB, M., RIBOLI, E., ROMAGUERA, D., MOUW, T. & GONZÃLEZ, C. A. (2009) Adherence to a Mediterranean diet and risk of gastric adenocarcinoma within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study. *The American Journal of Clinical Nutrition,* 91, 381-390.

BUONOCORE, S., AHERN, P. P., UHLIG, H. H., IVANOV, I. I., LITTMAN, D. R., MALOY, K. J. & POWRIE, F. (2010) Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature,* 464, 1371-5.

BURGLER, S., OUAKED, N., BASSIN, C., BASINSKI, T. M., MANTEL, P.-Y., SIEGMUND, K., MEYER, N., AKDIS, C. A. & SCHMIDT-WEBER, C. B. (2009) Differentiation and functional analysis of human TH17 cells. *Journal of Allergy and Clinical Immunology,* 123, 588-595.e7.

CACCAMO, N., LA MENDOLA, C., ORLANDO, V., MERAVIGLIA, S., TODARO, M., STASSI, G., SIRECI, G., FOURNIÃ, J. J. & DIELI, F. (2011) Differentiation, phenotype, and function of interleukin-17 producing human Vg9Vd2 T cells. *Blood,* 118, 129-138.

CAMERON, A. J., OTT, B. J. & PAYNE, W. S. (1985) The Incidence of Adenocarcinoma in Columnar-Lined (Barrett's) Esophagus. *New England Journal of Medicine,* 313, 857-859.

CANEDO, P., CORSO, G., PEREIRA, F., LUNET, N., SURIANO, G., FIGUEIREDO, C., PEDRAZZANI, C., MOREIRA, H., BARROS, H., CARNEIRO, F., SERUCA, R., ROVIELLO, F. & MACHADO, J. C. (2008) The interferon gamma receptor 1 (IFNGR1) -56C/T gene polymorphism is associated with increased risk of early gastric carcinoma. *Gut,* 57, 1504-8.

CANTOR, H. & SHINOHARA, M. L. (2009) Regulation of T-helper-cell lineage development by osteopontin: the inside story. *Nature Reviews Immunology,* 9, 137-41.

CARGILL, M., SCHRODI, S. J., CHANG, M., GARCIA, V. E., BRANDON, R., CALLIS, K. P., MATSUNAMI, N., ARDLIE, K. G., CIVELLO, D., CATANESE, J. J., LEONG, D. U., PANKO, J. M., MCALLISTER, L. B., HANSEN, C. B., PAPENFUSS, J., PRESCOTT, S. M., WHITE, T. J., LEPPERT, M. F., KRUEGER, G. G. & BEGOVICH, A. B. (2007) A Large-Scale Genetic Association Study Confirms IL12B and Leads to the Identification of IL23R as Psoriasis-Risk Genes. *American journal of human genetics,* 80, 273-290.

CARUSO, R., FINA, D., PAOLUZI, O. A., BLANCO, G. D. V., STOLFI, C., RIZZO, A., CAPRIOLI, F., SARRA, M., FABIO, A., FANTINI, M. C., MACDONALD, T. T., PALLONE, F. & MONTELEONE, G. (2008) IL-23-mediated regulation of IL-17 production in *Helicobacter pylori*-infected gastric mucosa. *European Journal of Immunology,* 38, 470-478.

CARUSO, R., FINA, D., PELUSO, I., FANTINI, M. C., TOSTI, C., DEL VECCHIO BLANCO, G., PAOLUZI, O. A., CAPRIOLI, F., ANDREI, F., STOLFI, C., ROMANO, M., RICCI, V., MACDONALD, T. T., PALLONE, F. & MONTELEONE, G. (2007a) IL-21 is highly produced in Helicobacter pylori-infected gastric mucosa and promotes gelatinases synthesis. *Journal of Immunology,* 178, 5957-65.

CARUSO, R., FINA, D., PELUSO, I., STOLFI, C., FANTINI, M. C., GIOIA, V., CAPRIOLI, F., DEL VECCHIO BLANCO, G., PAOLUZI, O. A., MACDONALD, T. T., PALLONE, F. & MONTELEONE, G. (2007b) A functional role for interleukin-21 in promoting the synthesis of the T-cell chemoattractant, MIP-3alpha, by gut epithelial cells. *Gastroenterology,* 132, 166-75.

CECCONI, V., MORO, M., DEL MARE, S., DELLABONA, P. & CASORATI, G. (2008) Use of MHC class II tetramers to investigate CD4+ T cell responses: problems and solutions. *Cytometry Part A: The Journal of the International Society for Analytical Cytology,* 73, 1010-8.

CELLA, M., OTERO, K. & COLONNA, M. (2010) Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proceedings of the National Academy of Sciences of the United States of America,* 107, 10961-6.

CHAN, W. Y., HUI, P. K., CHAN, J. K., CHEUNG, P. S., NG, C. S., SHAM, C. H. & GWI, E. (1991) Epithelial damage by Helicobacter pylori in gastric ulcers. *Histopathology,* 19, 47-53.

CHANG, Q., WANG, Y.-K., ZHAO, Q., WANG, C.-Z., HU, Y.-Z. & WU, B.-Y. (2012) Th17 cells are increased with severity of liver inflammation in patients with chronic hepatitis C. *Journal of Gastroenterology & Hepatology,* 27, 273-8.

CHANG, S. H., PARK, H. & DONG, C. (2006) Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. *Journal of Biological Chemistry,* 281, 35603-7.

CHATTOPADHYAY, P. K., YU, J. & ROEDERER, M. (2005) A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. *Nature Medicine,* 11, 1113-7.

CHAUHAN, S. K., EL ANNAN, J., ECOIFFIER, T., GOYAL, S., ZHANG, Q., SABAN, D. R. & DANA, R. (2009) Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. *Journal of Immunology,* 182, 1247-52.

CHEN, W., SHU, D. & CHADWICK, V. S. (2001) Helicobacter pylori infection: mechanism of colonization and functional dyspepsia Reduced colonization of gastric mucosa by Helicobacter pylori in mice deficient in interleukin-10. *Journal of gastroenterology and hepatology,* 16, 377-383.

CHEN, X., WAN, J., LIU, J., XIE, W., DIAO, X., XU, J., ZHU, B. & CHEN, Z. (2009) Increased IL-17-producing cells correlate with poor survival and lymphangiogenesis in NSCLC patients. *Lung Cancer,* 69, 348-54.

CHEN, Y. & BLASER, M. J. (2007) Inverse associations of Helicobacter pylori with asthma and allergy. *Archives of Internal Medicine,* 167, 821-7.

CHEN, Y., HAINES, C. J., GUTCHER, I., HOCHWELLER, K., BLUMENSCHEIN, W. M., MCCLANAHAN, T., HAMMERLING, G., LI, M. O., CUA, D. J. & MCGEACHY, M. J. (2011) Foxp3(+) regulatory T cells promote T helper 17 cell development in vivo through regulation of interleukin-2. *Immunity,* 34, 409-21.

CHIBA, T., MARUSAWA, H. & USHIJIMA, T. (2012) Inflammation-Associated Cancer Development in Digestive Organs: Mechanisms and Roles for Genetic and Epigenetic Modulation. *Gastroenterology,* 143, 550-563.

CODARRI, L., GYULVESZI, G., TOSEVSKI, V., HESSKE, L., FONTANA, A., MAGNENAT, L., SUTER, T. & BECHER, B. (2011) RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nature Immunology,* 12, 560-7.

COHEN, G. B., KAUR, A. & JOHNSON, R. P. (2005) Isolation of viable antigen-specific CD4 T cells by CD40L surface trapping. *Journal of Immunological Methods,* 302, 103-15.

CONTI, H. R., SHEN, F., NAYYAR, N., STOCUM, E., SUN, J. N., LINDEMANN, M. J., HO, A. W., HAI, J. H., YU, J. J., JUNG, J. W., FILLER, S. G., MASSO-WELCH, P., EDGERTON, M. & GAFFEN, S. L. (2009) Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *Journal of Experimental Medicine,* 206, 299-311.

COOMBES, J. L., SIDDIQUI, K. R. R., ARANCIBIA-CARCAMO, C. V., HALL, J., SUN, C.-M., BELKAID, Y. & POWRIE, F. (2007) A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *Journal of Experimental Medicine,* 204, 1757-64.

CORREA, P. (1988) A human model of gastric carcinogenesis. *Cancer Research,* 48, 3554-60.

COSMI, L., CIMAZ, R., MAGGI, L., SANTARLASCI, V., CAPONE, M., BORRIELLO, F., FROSALI, F., QUERCI, V., SIMONINI, G., BARRA, G., PICCINNI, M. P., LIOTTA, F., DE PALMA, R., MAGGI, E., ROMAGNANI, S. & ANNUNZIATO, F. (2011) Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the synovial fluid of patients with juvenile idiopathic arthritis. *Arthritis & Rheumatism,* 63, 2504-2515.

COSMI, L., DE PALMA, R., SANTARLASCI, V., MAGGI, L., CAPONE, M., FROSALI, F., RODOLICO, G., QUERCI, V., ABBATE, G., ANGELI, R., BERRINO, L., FAMBRINI, M., CAPRONI, M., TONELLI, F., LAZZERI, E., PARRONCHI, P., LIOTTA, F., MAGGI, E., ROMAGNANI, S. & ANNUNZIATO, F. (2008) Human interleukin-17-producing cells originate from a CD161+CD4+ T cell precursor. *The Journal of Experimental Medicine,* 205, 1903-1916.

COVER, T. L. & ATHERTON, J. C. (2009) Helicobacter pylori VacA toxin. IN PROFT, T. (Ed.) *Microbial Toxins: Current Research and Future Trends.* Horizon Scientific Press.

CRABTREE, J. E., SHALLCROSS, T. M., HEATLEY, R. V. & WYATT, J. I. (1991) Mucosal tumour necrosis factor alpha and interleukin-6 in patients with Helicobacter pylori associated gastritis. *Gut,* 32, 1473-7.

CROME, S. Q., WANG, A. Y., KANG, C. Y. & LEVINGS, M. K. (2009) The role of retinoic acid-related orphan receptor variant 2 and IL-17 in the development and function of human $CD4^+$ T cells. *European Journal of Immunology,* 39, 1480-93.

CROSS, A. J., FREEDMAN, N. D., REN, J., WARD, M. H., HOLLENBECK, A. R., SCHATZKIN, A., SINHA, R. & ABNET, C. C. (2010) Meat Consumption and Risk of Esophageal and Gastric Cancer in a Large Prospective Study. *Am J Gastroenterol.*

CUA, D. J., SHERLOCK, J., CHEN, Y., MURPHY, C. A., JOYCE, B., SEYMOUR, B., LUCIAN, L., TO, W., KWAN, S., CHURAKOVA, T., ZURAWSKI, S., WIEKOWSKI, M., LIRA, S. A., GORMAN, D., KASTELEIN, R. A. & SEDGWICK, J. D. (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature,* 421, 744-8.

CUA, D. J. & TATO, C. M. Innate IL-17-producing cells: the sentinels of the immune system. *Nature Reviews Immunology,* 10, 479-89.

CUI, G., HOUGHTON, J., FINKEL, N., CARLSON, J. & WANG, T. C. (2003) IFN-gamma infusion induces gastric atrophy, metaplasia and dysplasia in the absence of Helicobacter infection-a role for immune response in Helicobacter disease. *Gastroenterology,* 124, A19.

CUPEDO, T., CRELLIN, N. K., PAPAZIAN, N., ROMBOUTS, E. J., WEIJER, K., GROGAN, J. L., FIBBE, W. E., CORNELISSEN, J. J. & SPITS, H. (2009) Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nature Immunology,* 10, 66-74.

CURD, L. M., FAVORS, S. E. & GREGG, R. K. (2012) Pro-tumour activity of interleukin-22 in HPAFII human pancreatic cancer cells. *Clinical & Experimental Immunology,* 168, 192-9.

D'ELIOS, M. M., AMEDEI, A. & DEL PRETE, G. (2003) Helicobacter pylori antigen-specific T-cell responses at gastric level in chronic gastritis, peptic ulcer, gastric cancer and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma. *Microbes & Infection,* 5, 723-30.

D'ELIOS, M. M., MANGHETTI, M., DE CARLI, M., COSTA, F., BALDARI, C. T., BURRONI, D., TELFORD, J. L., ROMAGNANI, S. & DEL PRETE, G. (1997) T helper 1 effector cells specific for Helicobacter pylori in the gastric antrum of patients with peptic ulcer disease. *Journal of Immunology,* 158, 962-7.

DAMBACHER, J., BEIGEL, F., ZITZMANN, K., DE TONI, E. N., GOKE, B., DIEPOLDER, H. M., AUERNHAMMER, C. J. & BRAND, S. (2009) The role of the novel Th17 cytokine IL-26 in intestinal inflammation. *Gut,* 58, 1207-17.

DARDALHON, V., AWASTHI, A., KWON, H., GALILEOS, G., GAO, W., SOBEL, R. A., MITSDOERFFER, M., STROM, T. B., ELYAMAN, W., HO, I. C., KHOURY, S., OUKKA, M. & KUCHROO, V. K. (2008) IL-4 inhibits TGF-β-induced Foxp3+ T cells and, together with TGF-β, generates IL-9+ IL-10+ Foxp3- effector T cells. *Nat Immunol,* 9, 1347-1355.

DE BOLOS, C., GARRIDO, M. & REAL, F. X. (1995) MUC6 apomucin shows a distinct normal tissue distribution that correlates with Lewis antigen expression in the human stomach. *Gastroenterology,* 109, 723-734.

DE JONGE, R., KUIPERS, E. J., LANGEVELD, S. C. L., LOFFELD, R. J. L. F., STOOF, J., VAN VLIET, A. H. M. & KUSTERS, J. G. (2004) The Helicobacter pylori plasticity region locus jhp0947-jhp0949 is associated with duodenal ulcer disease and interleukin-12 production in monocyte cells. *FEMS Immunology & Medical Microbiology,* 41, 161-7.

DE MARTEL, C., FERLAY, J., FRANCESCHI, S., VIGNAT, J., BRAY, F., FORMAN, D. & PLUMMER, M. (2012) Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncology,* 13, 607-15.

DEAGLIO, S., DWYER, K. M., GAO, W., FRIEDMAN, D., USHEVA, A., ERAT, A., CHEN, J.-F., ENJYOJI, K., LINDEN, J., OUKKA, M., KUCHROO, V. K., STROM, T. B. & ROBSON, S. C.

(2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *Journal of Experimental Medicine,* 204, 1257-65.

DELYRIA, E. S., NEDRUD, J. G., ERNST, P. B., ALAM, M. S., REDLINE, R. W., DING, H., CZINN, S. J., XU, J. & BLANCHARD, T. G. (2011) Vaccine-induced immunity against Helicobacter pylori in the absence of IL-17A. *Helicobacter,* 16, 169-78.

DELYRIA, E. S., REDLINE, R. W. & BLANCHARD, T. G. (2009) Vaccination of mice against H pylori induces a strong Th-17 response and immunity that is neutrophil dependent. *Gastroenterology,* 136, 247-56.

DERHOVANESSIAN, E., ADAMS, V., HAHNEL, K., GROEGER, A., PANDHA, H., WARD, S. & PAWELEC, G. (2009) Pretreatment frequency of circulating IL-17+ CD4+ T-cells, but not Tregs, correlates with clinical response to whole-cell vaccination in prostate cancer patients. *International Journal of Cancer,* 125, 1372-9.

DI GENOVA, G., SAVELYEVA, N., SUCHACKI, A., THIRDBOROUGH, S. M. & STEVENSON, F. K. Bystander stimulation of activated CD4+ T cells of unrelated specificity following a booster vaccination with tetanus toxoid. *European Journal of Immunology,* 40, 976-85.

DI TOMMASO, A., XIANG, Z., BUGNOLI, M., PILERI, P., FIGURA, N., BAYELI, P. F., RAPPUOLI, R., ABRIGNANI, S. & DE MAGISTRIS, M. T. (1995) Helicobacter pylori-specific CD4+ T-cell clones from peripheral blood and gastric biopsies. *Infection & Immunity,* 63, 1102-6.

DISIS, M. L., DELA ROSA, C., GOODELL, V., KUAN, L.-Y., CHANG, J. C. C., KUUS-REICHEL, K., CLAY, T. M., KIM LYERLY, H., BHATIA, S., GHANEKAR, S. A., MAINO, V. C. & MAECKER, H. T. (2006) Maximizing the retention of antigen specific lymphocyte function after cryopreservation. *Journal of Immunological Methods,* 308, 13-8.

DIXON, M. F., GENTA, R. M., YARDLEY, J. H. & CORREA, P. (1996) Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *American Journal of Surgical Pathology,* 20, 1161-81.

DJOBA SIAWAYA, J. F., ROBERTS, T., BABB, C., BLACK, G., GOLAKAI, H. J., STANLEY, K., BAPELA, N. B., HOAL, E., PARIDA, S., VAN HELDEN, P. & WALZL, G. (2008) An Evaluation of Commercial Fluorescent Bead-Based Luminex Cytokine Assays. *PLoS ONE,* 3, e2535.

DO, J.-S., VISPERAS, A., DONG, C., BALDWIN, W. M., 3RD & MIN, B. (2011) Cutting edge: Generation of colitogenic Th17 CD4 T cells is enhanced by IL-17+ gammadelta T cells. *Journal of Immunology,* 186, 4546-50.

DOISNE, J.-M., BECOURT, C., AMNIAI, L., DUARTE, N., LE LUDUEC, J.-B. T., EBERL, G. R. & BENLAGHA, K. (2009) Skin and Peripheral Lymph Node Invariant NKT Cells Are Mainly Retinoic Acid Receptor-Related Orphan Receptor gt+ and Respond Preferentially under Inflammatory Conditions. *The Journal of Immunology,* 183, 2142-2149.

DU, M., DISS, T. C., XU, C., PENG, H., ISAACSON, P. G. & PAN, L. (1996) Ongoing mutation in MALT lymphoma immunoglobulin gene suggests that antigen stimulation plays a role in the clonal expansion. *Leukemia,* 10, 1190-7.

DUBIN, P. J., MCALLISTER, F. & KOLLS, J. K. (2007) Is cystic fibrosis a TH17 disease? *Inflammation Research,* 56, 221-7.

DUBINSKY, M. C., WANG, D., PICORNELL, Y., WROBEL, I., KATZIR, L., QUIROS, A., DUTRIDGE, D., WAHBEH, G., SILBER, G., BAHAR, R., MENGESHA, E., TARGAN, S. R., TAYLOR, K. D., ROTTER, J. I. & WESTERN REGIONAL RESEARCH ALLIANCE FOR PEDIATRIC, I. B. D. (2007) IL-23 receptor (IL-23R) gene protects against pediatric Crohn's disease. *Inflammatory Bowel Diseases,* 13, 511-5.

DUERR, R. H., TAYLOR, K. D., BRANT, S. R., RIOUX, J. D., SILVERBERG, M. S., DALY, M. J., STEINHART, A. H., ABRAHAM, C., REGUEIRO, M., GRIFFITHS, A., DASSOPOULOS, T., BITTON, A., YANG, H., TARGAN, S., DATTA, L. W., KISTNER, E. O., SCHUMM, L. P., LEE, A. T., GREGERSEN, P. K., BARMADA, M. M., ROTTER, J. I., NICOLAE, D. L. & CHO, J. H. (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science,* 314, 1461-3.

DUNN, B. E., CAMPBELL, G. P., PEREZ-PEREZ, G. I. & BLASER, M. J. (1990) Purification and characterization of urease from Helicobacter pylori. *Journal of Biological Chemistry,* 265, 9464-9469.

DUNN, B. E., COHEN, H. & BLASER, M. J. (1997) *Helicobacter pylori. Clinical Microbiology Reviews,* 10, 720-741.

DZIONEK, A., FUCHS, A., SCHMIDT, P., CREMER, S., ZYSK, M., MILTENYI, S., BUCK, D. W. & SCHMITZ, J. (2000) BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *Journal of Immunology,* 165, 6037-46.

EATON, K. A., BENSON, L. H., HAEGER, J. & GRAY, B. M. (2006) Role of Transcription Factor T-bet Expression by $CD4^+$ Cells in Gastritis Due to *Helicobacter pylori* in Mice. *Infection and Immunity,* 74, 4673-84.

EATON, K. A., MEFFORD, M. & THEVENOT, T. (2001) The role of T cell subsets and cytokines in the pathogenesis of Helicobacter pylori gastritis in mice. *Journal of Immunology,* 166, 7456-61.

EBERL, G., MARMON, S., SUNSHINE, M.-J., RENNERT, P. D., CHOI, Y. & LITTMAN, D. R. (2004) An essential function for the nuclear receptor ROR[gamma]t in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol,* 5, 64-73.

EL-BEHI, M., CIRIC, B., DAI, H., YAN, Y., CULLIMORE, M., SAFAVI, F., ZHANG, G.-X., DITTEL, B. N. & ROSTAMI, A. (2011) The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nature Immunology,* 12, 568-75.

EL-BEHI, M., CIRIC, B., YU, S., ZHANG, G.-X., FITZGERALD, D. C. & ROSTAMI, A. (2009) Differential Effect of IL-27 on Developing versus Committed Th17 Cells. *The Journal of Immunology,* 183, 4957-4967.

EL-OMAR, E., RABKIN, C., GAMMON, M., VAUGHAN, T., RISCH, H., SCHOENBERG, J., STANFORD, J., MAYNE, S., GOEDERT, J., BLOT, W., FRAUMENI, J. J. & CHOW, W. (2003) Increased Risk of Noncardia Gastric Cancer Associated With Proinflammatory Cytokine Gene Polymorphisms. *Gastroenterology,* 124, 1193-201.

EL-OMAR, E. M., CARRINGTON, M., CHOW, W.-H., MCCOLL, K. E. L., BREAMK, J. H., YOUNGK, H. A., HERRERA, J., LISSOWSKA, J., YUAN, C.-C., ROTHMAN, N., LANYON, G., MARTIN, M., FRAUMENI JR, J. F. & RABKIN, C. S. (2000) Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature,* 404, 398-402.

EL-OMAR, E. M., NG, M. T. & HOLD, G. L. (2008) Polymorphisms in Toll-like receptor genes and risk of cancer. *Oncogene,* 27, 244-52.

EL MIEDANY, Y. M., BADDOUR, M., AHMED, I. & FAHMY, H. (2005) Sjogren's syndrome: concomitant H. Pylori infection and possible correlation with clinical parameters. *Joint Bone Spine,* 72, 135-141.

ENARSSON, K., LUNDGREN, A., KINDLUND, B., HERMANSSON, M., RONCADOR, G., BANHAM, A. H., LUNDIN, B. S. & QUIDING-JARBRINK, M. (2006) Function and recruitment of mucosal regulatory T cells in human chronic *Helicobacter pylori* infection and gastric adenocarcinoma. *Clinical Immunology,* 121, 358-68.

ERMAK, T. H., GIANNASCA, P. J., NICHOLS, R., MYERS, G. A., NEDRUD, J., WELTZIN, R., LEE, C. K., KLEANTHOUS, H. & MONATH, T. P. (1998) Immunization of mice with urease vaccine affords protection against Helicobacter pylori infection in the absence of antibodies and is mediated by MHC class II-restricted responses. *Journal of Experimental Medicine,* 188, 2277-88.

EYERICH, K., FOERSTER, S., ROMBOLD, S., SEIDL, H.-P., BEHRENDT, H., HOFMANN, H., RING, J. & TRAIDL-HOFFMANN, C. (2008) Patients with chronic mucocutaneous candidiasis exhibit reduced production of Th17-associated cytokines IL-17 and IL-22. *Journal of Investigative Dermatology,* 128, 2640-5.

EYERICH, S., EYERICH, K., PENNINO, D., CARBONE, T., NASORRI, F., PALLOTTA, S., CIANFARANI, F., ODORISIO, T., TRAIDL-HOFFMANN, C., BEHRENDT, H., DURHAM, S. R., SCHMIDT-WEBER, C. B. & CAVANI, A. (2009) Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *Journal of Clinical Investigation,* 119, 3573-85.

EZRA J. BARZILAY & FAGAN, R. P. (2012) Helicobacter pylori. *http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/helicobacter-pylori.htm. Accessed 20/1/13.* Centres for Disease Control and Prevention.

FAN, X. J., CHUA, A., SHAHI, C. N., MCDEVITT, J., KEELING, P. W. & KELLEHER, D. (1994) Gastric T lymphocyte responses to Helicobacter pylori in patients with H pylori colonisation. *Gut,* 35, 1379-84.

FANTINI, M. C., MONTELEONE, G. & MACDONALD, T. T. (2008) IL-21 comes of age as a regulator of effector T cells in the gut. *Mucosal immunology,* 1, 110-5.

FASSBENDER, K., RAGOSCHKE, A., ROSSOL, S., SCHWARTZ, A., MIELKE, O., PAULIG, A. & HENNERICI, M. (1998) Increased release of interleukin-12p40 in MS: association with intracerebral inflammation. *Neurology,* 51, 753-8.

FEHLINGS, M., DROBBE, L., MOOS, V., RENNER VIVEROS, P., HAGEN, J., BEIGIER-BOMPADRE, M., PANG, E., BELOGOLOVA, E., CHURIN, Y., SCHNEIDER, T., MEYER, T. F., AEBISCHER, T. & IGNATIUS, R. (2012) Comparative Analysis of the Interaction of Helicobacter pylori with Human Dendritic Cells, Macrophages, and Monocytes. *Infection & Immunity,* 80, 2724-34.

FENOGLIO, D., POGGI, A., CATELLANI, S., BATTAGLIA, F., FERRERA, A., SETTI, M., MURDACA, G. & ZOCCHI, M. R. (2009) Vdelta1 T lymphocytes producing IFN-gamma and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans. *Blood,* 113, 6611-8.

FERREIRA, R. M., MACHADO, J. C., LEITE, M., CARNEIRO, F. & FIGUEIREDO, C. (2012) The number of Helicobacter pylori CagA EPIYA C tyrosine phosphorylation motifs influences the pattern of gastritis and the development of gastric carcinoma. *Histopathology,* 60, 992-998.

FERWERDA, B., FERWERDA, G., PLANTINGA, T. S., WILLMENT, J. A., VAN SPRIEL, A. B., VENSELAAR, H., ELBERS, C. C., JOHNSON, M. D., CAMBI, A., HUYSAMEN, C., JACOBS, L., JANSEN, T., VERHEIJEN, K., MASTHOFF, L., MORRE, S. A., VRIEND, G., WILLIAMS, D. L., PERFECT, J. R., JOOSTEN, L. A. B., WIJMENGA, C., VAN DER MEER, J. W. M., ADEMA, G. J., KULLBERG, B. J., BROWN, G. D. & NETEA, M. G. (2009) Human dectin-1 deficiency and mucocutaneous fungal infections. *New England Journal of Medicine,* 361, 1760-7.

FIGUEIREDO, C., MACHADO, J., PHAROAH, P., SERUCA, R., SOUSA, S., CARVALHO, R., CAPELINHA, A., QUINT, W., CALDAS, C., VAN DOORN, L., CARNEIRO, F. & M., S.-S. (2002) Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. *Journal of the National Cancer Institute,* 94, 1680-7.

FINA, D., SARRA, M., CARUSO, R., DEL VECCHIO BLANCO, G., PALLONE, F., MACDONALD, T. T. & MONTELEONE, G. (2008) Interleukin 21 contributes to the mucosal T helper cell type 1 response in coeliac disease. *Gut,* 57, 887-92.

FITZGERALD, D. C., ZHANG, G.-X., EL-BEHI, M., FONSECA-KELLY, Z., LI, H., YU, S., SARIS, C. J. M., GRAN, B., CIRIC, B. & ROSTAMI, A. (2007) Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27–stimulated T cells. *Nature Immunology,* 8, 1372-9.

FLACH, C.-F., OSTBERG, A. K., NILSSON, A.-T., MALEFYT, R. D. W. & RAGHAVAN, S. Proinflammatory cytokine gene expression in the stomach correlates with vaccine-induced protection against Helicobacter pylori infection in mice: an important role for interleukin-17 during the effector phase. *Infection & Immunity,* 79, 879-86.

FLETCHER, J. M., LONERGAN, R., COSTELLOE, L., KINSELLA, K., MORAN, B., O'FARRELLY, C., TUBRIDY, N. & MILLS, K. H. G. (2009) CD39+Foxp3+ Regulatory T Cells Suppress Pathogenic Th17 Cells and Are Impaired in Multiple Sclerosis. *The Journal of Immunology,* 183, 7602-7610.

FOCK, K. M., KATELARIS, P., SUGANO, K., ANG, T. L., HUNT, R., TALLEY, N. J., LAM, S. K., XIAO, S.-D., TAN, H. J., WU, C.-Y., JUNG, H. C., HOANG, B. H., KACHINTORN, U., GOH, K.-L., CHIBA, T., RANI, A. A. & SECOND ASIA-PACIFIC, C. (2009) Second Asia-Pacific Consensus Guidelines for Helicobacter pylori infection. *Journal of Gastroenterology & Hepatology,* 24, 1587-600.

FONTENOT, J. D., GAVIN, M. A. & RUDENSKY, A. Y. (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunology,* 4, 330-6.

FOX, J. G., BECK, P., DANGLER, C. A., WHARY, M. T., WANG, T. C., SHI, H. N. & NAGLER-ANDERSON, C. (2000) Concurrent enteric helminth infection modulates

inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. *Nature medicine,* 6, 536-42.

FOX, J. G. & WANG, T. C. (2007) Inflammation, atrophy and gastric cancer. *The Journal of Clinical Investigation,* 117, 60-69.

FRANK, K. M., ZHOU, T., MORENO-VINASCO, L., HOLLETT, B., GARCIA, J. G. N. & BUBECK WARDENBURG, J. (2012) Host response signature to Staphylococcus aureus alpha-hemolysin implicates pulmonary Th17 response. *Infection & Immunity,* 80, 3161-9.

FREIRE DE MELO, F. C., ROCHA, A. M. C., ROCHA, G. A., PEDROSO, S. H. S. P., DE ASSIS BATISTA, S. R., FONSECA DE CASTRO, L. C. P., CARVALHO, S. D., BITTENCOURT, P. F. S., DE OLIVEIRA, C. A., CORRÃªA-OLIVEIRA, R. & MAGALHÃ£ES QUEIROZ, D. M. A regulatory instead of an IL-17 T response predominates in Helicobacter pylori-associated gastritis in children. *Microbes and Infection,* 14, 341-347.

FRENTSCH, M., ARBACH, O., KIRCHHOFF, D., MOEWES, B., WORM, M., ROTHE, M., SCHEFFOLD, A. & THIEL, A. (2005) Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. *Nature Medicine,* 11, 1118-24.

FUTAGAMI, S., HIRATSUKA, T., SUZUKI, K., KUSUNOKI, M., WADA, K., MIYAKE, K., OHASHI, K., SHIMIZU, M., TAKAHASHI, H., GUDIS, K., KATO, S., TSUKUI, T. & SAKAMOTO, C. (2006) gammadelta T cells increase with gastric mucosal interleukin (IL)-7, IL-1beta, and Helicobacter pylori urease specific immunoglobulin levels via CCR2 upregulation in Helicobacter pylori gastritis. *Journal of Gastroenterology & Hepatology,* 21, 32-40.

GAFFEN, S. L. (2008) An overview of IL-17 function and signaling *Cytokine.*

GAFFEN, S. L. (2011) Recent advances in the IL-17 cytokine family. *Current Opinion in Immunology,* 23, 613-9.

GALGANI, M., BUSIELLO, I., CENSINI, S., ZAPPACOSTA, S., RACIOPPI, L. & ZARRILLI, R. (2004) Helicobacter pylori induces apoptosis of human monocytes but not monocyte-derived dendritic cells: role of the cag pathogenicity island. *Infection & Immunity,* 72, 4480-5.

GALMICHE, A., RASSOW, J., DOYE, A., CAGNOL, S., CHAMBARD, J.-C., CONTAMIN, S., DE THILLOT, V., JUST, I., RICCI, V., SOLCIA, E., OBBERGHEN, E. V. & BOQUET, P. (2000) The N-terminal 34 kDa fragment of Helicobacter pylori vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO,* 19, 6361-70.

GAO, L., MICHEL, A., WECK, M. N., ARNDT, V., PAWLITA, M. & BRENNER, H. (2009) Helicobacter pylori Infection and Gastric Cancer Risk: Evaluation of 15 H. pylori Proteins Determined by Novel Multiplex Serology. *Cancer Research,* 69, 6164-6170.

GARCIA-HERNANDEZ, M. D. L. L., HAMADA, H., REOME, J. B., MISRA, S. K., TIGHE, M. P. & DUTTON, R. W. (2010) Adoptive transfer of tumor-specific Tc17 effector T cells controls the growth of B16 melanoma in mice. *Journal of Immunology,* 184, 4215-27.

GARHART, C. A., HEINZEL, F. P., CZINN, S. J. & NEDRUD, J. G. (2003) Vaccine-induced reduction of Helicobacter pylori colonization in mice is interleukin-12 dependent but gamma interferon and inducible nitric oxide synthase independent. *Infection & Immunity,* 71, 910-21.

GE, J., WANG, K., MENG, Q.-H., QI, Z.-X., MENG, F.-L. & FAN, Y.-C. (2009) Implication of Th17 and Th1 cells in patients with chronic active hepatitis B. *Journal of Clinical Immunology,* 30, 60-7.

GEBERT, B., FISCHER, W., WEISS, E., HOFFMANN, R. & HAAS, R. (2003) Helicobacter pylori Vacuolating Cytotoxin Inhibits T Lymphocyte Activation. *Science,* 301, 1099-102.

GENOVESE, M. C., VAN DEN BOSCH, F., ROBERSON, S. A., BOJIN, S., BIAGINI, I. M., RYAN, P. & SLOAN-LANCASTER, J. (2010) LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: A phase I randomized, double-blind, placebo-controlled, proof-of-concept study. *Arthritis & Rheumatism,* 62, 929-39.

GEWIRTZ, A. T., YU, Y., KRISHNA, U. S., ISRAEL, D. A., LYONS, S. L. & PEEK, R. M., JR. (2004) Helicobacter pylori flagellin evades toll-like receptor 5-mediated innate immunity. *Journal of Infectious Diseases,* 189, 1914-20.

GHIRINGHELLI, F., MENARD, C., MARTIN, F. & ZITVOGEL, L. (2006) The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. *Immunological Reviews,* 214, 229-238.

GHORESCHI, K., LAURENCE, A., YANG, X.-P., TATO, C. M., MCGEACHY, M. J., KONKEL, J. E., RAMOS, H. L., WEI, L., DAVIDSON, T. S., BOULADOUX, N., GRAINGER, J. R., CHEN, Q., KANNO, Y., WATFORD, W. T., SUN, H.-W., EBERL, G., SHEVACH, E. M., BELKAID, Y., CUA, D. J., CHEN, W. & O'SHEA, J. J. (2010) Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature,* 467, 967-71.

GIULIETTI, A., OVERBERGH, L., VALCKX, D., DECALLONNE, B., BOUILLON, R. & MATHIEU, C. (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods (Duluth),* 25, 386-401.

GLOCKER, E.-O., HENNIGS, A., NABAVI, M., SCHAFFER, A. A., WOELLNER, C., SALZER, U., PFEIFER, D., VEELKEN, H., WARNATZ, K., TAHAMI, F., JAMAL, S., MANGUIAT, A., REZAEI, N., AMIRZARGAR, A. A., PLEBANI, A., HANNESSCHLAGER, N., GROSS, O., RULAND, J. & GRIMBACHER, B. (2009) A homozygous CARD9 mutation in a family with susceptibility to fungal infections. *New England Journal of Medicine,* 361, 1727-35.

GNERLICH, J. L., MITCHEM, J. B., WEIR, J. S., SANKPAL, N. V., KASHIWAGI, H., BELT, B. A., POREMBKA, M. R., HERNDON, J. M., EBERLEIN, T. J., GOEDEGEBUURE, P. & LINEHAN, D. C. (2010) Induction of Th17 cells in the tumor microenvironment improves survival in a murine model of pancreatic cancer. *Journal of Immunology,* 185, 4063-71.

GOBERT, A. P., BAMBOU, J.-C., WERTS, C., BALLOY, V., CHIGNARD, M., MORAN, A. P. & FERRERO, R. L. (2004) Helicobacter pylori heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. *Journal of Biological Chemistry,* 279, 245-50.

GOH, K.-L., CHAN, W.-K., SHIOTA, S. & YAMAOKA, Y. (2011) Epidemiology of Helicobacter pylori Infection and Public Health Implications. *Helicobacter,* 16, 1-9.

GONZALEZ, C. A. & RIBOLI, E. (2010) Diet and cancer prevention: Contributions from the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *European Journal of Cancer,* 46, 2555-2562.

GOODWIN, C. S., ARMSTRONG, J. A., CHILVERS, T., PETERS, M., COLLINS, M. D., SLY, L., MCCONNELL, W. & HARPER, W. E. S. (1989) Transfer of Campylobacter pylori and Campylobacter mustelae to Helicobacter gen. nov. as Helicobacter pylori comb. nov. and Helicobacter mustelae comb. nov., Respectively. *International Journal of Systematic Bacteriology,* 39, 397-405.

GOOZ, M., SHAKER, M., GOOZ, P. & SMOLKA, A. J. (2003) Interleukin 1beta induces gastric epithelial cell matrix metalloproteinase secretion and activation during Helicobacter pylori infection. *Gut,* 52, 1250-6.

GRABOWSKA, A. M., LECHNER, F., KLENERMAN, P., TIGHE, P. J., RYDER, S., BALL, J. K., THOMSON, B. J., IRVING, W. L. & ROBINS, R. A. (2001) Direct ex vivo comparison of the breadth and specificity of the T cells in the liver and peripheral blood of patients with chronic HCV infection. *European Journal of Immunology,* 31, 2388-94.

GRAFMUELLER, S., BILLERBECK, E., BLUM, H. E., NEUMANN-HAEFELIN, C. & THIMME, R. (2012) Differential antigen specificity of hepatitis C virus-specific interleukin 17- and interferon gamma-producing CD8(+) T cells during chronic infection. *Journal of Infectious Diseases,* 205, 1142-6.

GRAHAM, D. Y. & GISBERT, J. P. (2013) Helicobacter pylori: Tailored therapy with novel sequential quadruple therapies. *Nat Rev Gastroenterol Hepatol,* 10, 6-8.

GRAN, B., ZHANG, G.-X., YU, S., LI, J., CHEN, X.-H., VENTURA, E. S., KAMOUN, M. & ROSTAMI, A. (2002) IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. *Journal of Immunology,* 169, 7104-10.

GREENAWAY, A., WINTER, J., HUSSAIN, K., LETLEY, D., STAPLES, E., ATHERTON, J. C. & ROBINSON, K. (2011) The Peripheral Blood Regulatory T Cell Response to Helicobacter Pylori Infection as a Marker for Peptic Ulcer Disease. *ICMI 11 Abstract supplement.*

http://www.socmucimm.org/images/icmi2011/abstract%20supplement%20-%20chronological%207-18-2011.pdf. Accessed 12/2/13.

GRUBMAN, A., KAPARAKIS, M., VIALA, J., ALLISON, C., BADEA, L., KARRAR, A., BONECA, I. G., LE BOURHIS, L., REEVE, S., SMITH, I. A., HARTLAND, E. L., PHILPOTT, D. J. & FERRERO, R. L. (2010) The innate immune molecule, NOD1, regulates direct killing of Helicobacter pylori by antimicrobial peptides. *Cellular Microbiology,* 12, 626-39.

GU, F.-M., LI, Q.-L., GAO, Q., JIANG, J.-H., ZHU, K., HUANG, X.-Y., PAN, J.-F., YAN, J., HU, J.-H., WANG, Z., DAI, Z., FAN, J. & ZHOU, J. (2011) IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma. *Molecular Cancer,* 10, 150.

GUINEY, D. G., HASEGAWA, P. & COLE, S. P. (2003) Helicobacter pylori preferentially induces interleukin 12 (IL-12) rather than IL-6 or IL-10 in human dendritic cells. *Infection & Immunity,* 71, 4163-6.

GUTCHER, I., DONKOR, M. K., MA, Q., RUDENSKY, A. Y., FLAVELL, R. A. & LI, M. O. (2011) Autocrine transforming growth factor-beta1 promotes in vivo Th17 cell differentiation. *Immunity,* 34, 396-408.

HAFSI, N., VOLAND, P., SCHWENDY, S., RAD, R., REINDL, W., GERHARD, M. & PRINZ, C. (2004) Human dendritic cells respond to Helicobacter pylori, promoting NK cell and Th1-effector responses in vitro. *Journal of Immunology,* 173, 1249-57.

HAMADA, H., GARCIA-HERNANDEZ, M. D. L. L., REOME, J. B., MISRA, S. K., STRUTT, T. M., MCKINSTRY, K. K., COOPER, A. M., SWAIN, S. L. & DUTTON, R. W. (2009) Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. *Journal of Immunology,* 182, 3469-81.

HANSSON, L.-E., NYREN, O., HSING, A. W., BERGSTROM, R., JOSEFSSON, S., CHOW, W.-H., FRAUMENI, J. F. & HANS-OLOV, A. (1996) The risk of stomach cancer in patients with gastric or duodenal ulcer disease. *New England Journal of Medicine,* 335, 242-9.

HANSSON, M., LUNDGREN, A., ELGBRATT, K., QUIDING-JARBRINK, M., SVENNERHOLM, A.-M. & JOHANSSON, E.-L. (2006) Dendritic cells express CCR7 and migrate in response to CCL19 (MIP-3beta) after exposure to Helicobacter pylori. *Microbes & Infection,* 8, 841-50.

HARRINGTON, L. E., HATTON, R. D., MANGAN, P. R., TURNER, H., MURPHY, T. L., MURPHY, K. M. & WEAVER, C. T. (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology,* 6, 1123-32.

HARRIS, P. R., MOBLEY, H. L., PEREZ-PEREZ, G. I., BLASER, M. J. & SMITH, P. D. (1996) Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. *Gastroenterology,* 111, 419-25.

HARRIS, P. R., WRIGHT, S. W., SERRANO, C., RIERA, F., DUARTE, I., TORRES, J., PENA, A., ROLLAN, A., VIVIANI, P., GUIRALDES, E., SCHMITZ, J. M., LORENZ, R. G., NOVAK, L., SMYTHIES, L. E. & SMITH, P. D. (2008) Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. *Gastroenterology,* 134, 491-9.

HASE, K., MURAKAMI, M., IIMURA, M., COLE, S. P., HORIBE, Y., OHTAKE, T., OBONYO, M., GALLO, R. L., ECKMANN, L. & KAGNOFF, M. F. (2003) Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against Helicobacter pylori. *Gastroenterology,* 125, 1613-1625.

HATZ, R. A., MEIMARAKIS, G., BAYERDÃRFFER, E., STOLTE, M., KIRCHNER, T. & ENDERS, G. (1996) Characterization of Lymphocytic Infiltrates in Helicobacter pylori-Associated Gastritis. *Scandinavian Journal of Gastroenterology,* 31, 222-228.

HAYASHI, S., SUGIYAMA, T., YOKOTA, K., ISOGAI, H., ISOGAI, E., OGUMA, K., ASAKA, M., FUJII, N. & HIRAI, Y. (1998) Analysis of immunoglobulin A antibodies to Helicobacter pylori in serum and gastric juice in relation to mucosal inflammation. *Clinical & Diagnostic Laboratory Immunology,* 5, 617-21.

HE, D., LI, H., YUSUF, N., ELMETS, C. A., LI, J., MOUNTZ, J. D. & XU, H. (2010) IL-17 promotes tumor development through the induction of tumor promoting microenvironments at tumor sites and myeloid-derived suppressor cells. *Journal of Immunology,* 184, 2281-8.

HE, X. X., YANG, J., DING, Y. W., LIU, W., SHEN, Q. Y. & XIA, H. H. X. (2006) Increased epithelial and serum expression of macrophage migration inhibitory factor (MIF) in gastric cancer: potential role of MIF in gastric carcinogenesis. *Gut,* 55, 797-802.

HEINZEL, F. P., HUJER, A. M., AHMED, F. N. & RERKO, R. M. (1997) In vivo production and function of IL-12 p40 homodimers. *Journal of Immunology,* 158, 4381-8.

HIGASHI, H., TSUTSUMI, R., MUTO, S., SUGIYAMA, T., AZUMA, T., ASAKA, M. & HATAKEYAMA, M. (2002) SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. *Science,* 295, 683-6.

HINRICHS, C. S., KAISER, A., PAULOS, C. M., CASSARD, L., SANCHEZ-PEREZ, L., HEEMSKERK, B., WRZESINSKI, C., BORMAN, Z. A., MURANSKI, P. & RESTIFO, N. P. (2009) Type 17 CD8+ T cells display enhanced antitumor immunity. *Blood,* 114, 596-9.

HIROTA, K., DUARTE, J. H., VELDHOEN, M., HORNSBY, E., LI, Y., CUA, D. J., AHLFORS, H., WILHELM, C., TOLAINI, M., MENZEL, U., GAREFALAKI, A., POTOCNIK, A. J. & STOCKINGER, B. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nature Immunology,* 12, 255-63.

HIROTA, K., TURNER, J.-E., VILLA, M., DUARTE, J. H., DEMENGEOT, J., STEINMETZ, O. M. & STOCKINGER, B. (2013) Plasticity of TH17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nat Immunol,* 14, 372-379.

HITZLER, I., KOHLER, E., ENGLER, D. B., YAZGAN, A. S. & MULLER, A. (2012a) The role of Th cell subsets in the control of Helicobacter infections and in T cell-driven gastric immunopathology. *Frontiers in Immunology,* 3, 142.

HITZLER, I., SAYI, A., KOHLER, E., ENGLER, D. B., KOCH, K. N., HARDT, W.-D. & MULLER, A. (2012b) Caspase-1 has both proinflammatory and regulatory properties in Helicobacter infections, which are differentially mediated by its substrates IL-1beta and IL-18. *Journal of Immunology,* 188, 3594-602.

HO, A. W., SHEN, F., CONTI, H. R., PATEL, N., CHILDS, E. E., PETERSON, A. C., HERNANDEZ-SANTOS, N., KOLLS, J. K., KANE, L. P., OUYANG, W. & GAFFEN, S. L. IL-17RC is required for immune signaling via an extended SEF/IL-17R signaling domain in the cytoplasmic tail. *Journal of Immunology,* 185, 1063-70.

HOEVE, M. A., SAVAGE, N. D. L., BOER, T. D., LANGENBERG, D. M. L., MALEFYT, R. D. W., OTTENHOFF, T. H. M. & VERRECK, F. A. W. (2006) Divergent effects of IL-12 and IL-23 on the production of IL-17 by human T cells. *European Journal of Immunology,* 36, 661-70.

HOFFMANN, F., ALBERT, M. H., ARENZ, S., BIDLINGMAIER, C., BERKOWICZ, N., SEDLACZEK, S., TILL, H., PAWLITA, I., RENNER, E. D., WEISS, M. & BELOHRADSKY, B. H. (2005) Intracellular T-cell cytokine levels are age-dependent in healthy children and adults. *European Cytokine Network,* 16, 283-8.

HOLCK, S., NORGAARD, A., BENNEDSEN, M., PERMIN, H., NORN, S. & ANDERSEN, L. P. (2003) Gastric mucosal cytokine responses in Helicobacter pylori-infected patients with gastritis and peptic ulcers. Association with inflammatory parameters and bacteria load. *FEMS Immunology & Medical Microbiology,* 36, 175-80.

HOLD, G. L., RABKIN, C. S., CHOW, W.-H., SMITH, M. G., GAMMON, M. D., RISCH, H. A., VAUGHAN, T. L., MCCOLL, K. E. L., LISSOWSKA, J., ZATONSKI, W., SCHOENBERG, J. B., BLOT, W. J., MOWAT, N. A. G., FRAUMENI, J. F., JR. & EL-OMAR, E. M. (2007) A functional polymorphism of toll-like receptor 4 gene increases risk of gastric carcinoma and its precursors. *Gastroenterology,* 132, 905-12.

HORI, S., NOMURA, T. & SAKAGUCHI, S. (2003) Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science,* 299, 1057-61.

HORVATH, D. J., WASHINGTON, M. K., COPE, V. A. & SCOTT ALGOOD, H. M. (2012) IL-23 contributes to control of chronic Helicobacter pylori infection and the development of T helper responses in a mouse model. *Frontiers in Immunology,* 3.

HOU, L., EL-OMAR, E. M., CHEN, J., GRILLO, P., RABKIN, C. S., BACCARELLI, A., YEAGER, M., CHANOCK, S. J., ZATONSKI, W., SOBIN, L. H., LISSOWSKA, J., FRAUMENI, J. F., JR. & CHOW, W. H. (2007) Polymorphisms in Th1-type cell-mediated response genes and risk of gastric cancer. *Carcinogenesis,* 28, 118-23.

HOUGHTON, J., MACERA-BLOCH, L. S., HARRISON, L., KIM, K. H. & KORAH, R. M. (2000) Tumor necrosis factor alpha and interleukin 1beta up-regulate gastric mucosal Fas antigen expression in Helicobacter pylori infection. *Infection & Immunity,* 68, 1189-95.

HOUGHTON, J. & WANG, T. C. (2005) Helicobacter pylori and Gastric Cancer: A New Paradigm For Inflammation-Associated Epithelial Cancers *Gastroenterology,* 128, 1567-78.

HSU, P.-I., JWO, J.-J., YANG, C.-L., HSU, P.-N., YANG, H.-B., LAI, K.-H., CHEN, I. S., CHUAH, S.-K., WU, D.-C. & CHEN, A. (2008a) Association of the myeloperoxidase polymorphism with the risk of gastric cancer. *Anticancer Research,* 28, 1317-23.

HSU, P.-I., LU, P.-J., WANG, E. M., GER, L.-P., LO, G.-H., TSAY, F.-W., CHEN, T.-A., YANG, H.-B., CHEN, H.-C., LIN, W.-S. & LAI, K.-H. (2008b) Polymorphisms of death pathway genes FAS and FASL and risk of premalignant gastric lesions. *Anticancer Research,* 28, 97-103.

HSU, P.-I., WU, D.-C., WU, J.-Y. & GRAHAM, D. Y. (2011) Modified Sequential Helicobacter pylori Therapy: Proton Pump Inhibitor and Amoxicillin for 14 Days with Clarithromycin and Metronidazole added as a Quadruple (Hybrid) Therapy for the Final 7 Days. *Helicobacter,* 16, 139-145.

HU, B., EL HAJJ, N., SITTLER, S., LAMMERT, N., BARNES, R. & MELONI-EHRIG, A. (2012) Gastric cancer: Classification, histology and application of molecular pathology. *Journal of gastrointestinal oncology,* 3, 251-261.

HUANG, Q., LIU, D., MAJEWSKI, P., SCHULTE, L. C., KORN, J. M., YOUNG, R. A., LANDER, E. S. & HACOHEN, N. (2001) The plasticity of dendritic cell responses to pathogens and their components. *Science,* 294, 870-5.

HUANG, X., QU, X., YAN, W., HUANG, Y., CAI, M., HU, B., WU, L., LIN, H., CHEN, Z., ZHU, C., LU, L., SUN, X., RONG, L., JIANG, Y., SUN, D., ZHONG, L. & XIONG, P. (2010) Iron deficiency anaemia can be improved after eradication of Helicobacter pylori. *Postgraduate Medical Journal,* 86, 272-8.

HUBER, M., HEINK, S., GROTHE, H., GURALNIK, A., REINHARD, K., ELFLEIN, K., HUNIG, T., MITTRUCKER, H.-W., BRUSTLE, A., KAMRADT, T. & LOHOFF, M. (2009) A Th17-like developmental process leads to CD8(+) Tc17 cells with reduced cytotoxic activity. *European Journal of Immunology,* 39, 1716-25.

HUEBER, W., PATEL, D. D., DRYJA, T., WRIGHT, A. M., KOROLEVA, I., BRUIN, G., ANTONI, C., DRAELOS, Z., GOLD, M. H., PSORIASIS STUDY, G., DUREZ, P., TAK, P. P., GOMEZ-REINO, J. J., RHEUMATOID ARTHRITIS STUDY, G., FOSTER, C. S., KIM, R. Y., SAMSON, C. M., FALK, N. S., CHU, D. S., CALLANAN, D., NGUYEN, Q. D., UVEITIS STUDY, G., ROSE, K., HAIDER, A. & DI PADOVA, F. (2010) Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis, rheumatoid arthritis, and uveitis. *Science Translational Medicine,* 2, 52ra72.

HUEBER, W., SANDS, B. E., LEWITZKY, S., VANDEMEULEBROECKE, M., REINISCH, W., HIGGINS, P. D. R., WEHKAMP, J., FEAGAN, B. G., YAO, M. D., KARCZEWSKI, M., KARCZEWSKI, J., PEZOUS, N., BEK, S., BRUIN, G., MELLGARD, B., BERGER, C., LONDEI, M., BERTOLINO, A. P., TOUGAS, G., TRAVIS, S. P. L. & FOR THE SECUKINUMAB IN CROHN'S DISEASE STUDY, G. (2012) Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut,* 61, 1693-1700.

HUH, J. R., LEUNG, M. W. L., HUANG, P., RYAN, D. A., KROUT, M. R., MALAPAKA, R. R. V., CHOW, J., MANEL, N., CIOFANI, M., KIM, S. V., CUESTA, A., SANTORI, F. R., LAFAILLE, J. J., XU, H. E., GIN, D. Y., RASTINEJAD, F. & LITTMAN, D. R. (2011) Digoxin and its derivatives suppress TH17 cell differentiation by antagonizing RORgammat activity. *Nature,* 472, 486-90.

HUSSAIN, K. (2012) PhD thesis: The Helicobacter pylori infection mediated regulatory T-cell response and in vivo suppression of allergy and autoimmunity. University of Nottingham.

HUSSAIN, S. P., HOFSETH, L. J. & HARRIS, C. C. (2003) Radical causes of cancer. *Nature Reviews Cancer,* 3, 276-85.

HUSSEIN, N. R., ARGENT, R. H., MARX, C. K., PATEL, S. R., ROBINSON, K. & ATHERTON, J. C. (2010) Helicobacter pylori dupA is polymorphic, and its active form induces

proinflammatory cytokine secretion by mononuclear cells. *Journal of Infectious Diseases,* 202, 261-9.

HWANG, I.-R., KODAMA, T., KIKUCHI, S., SAKAI, K., PETERSON, L. E., GRAHAM, D. Y. & YAMAOKA, Y. (2002) Effect of interleukin 1 polymorphisms on gastric mucosal interleukin 1b production in Helicobacter pylori infection. *Gastroenterology,* 123, 1793-1803.

IARC (1994) Volume 61: Schistosomes, Liver Flukes and Helicobacter pylori. *IARC monographs on the evaluation of carcinogenic risk to humans.* Lyon, France.

IIDA, T., IWAHASHI, M., KATSUDA, M., ISHIDA, K., NAKAMORI, M., NAKAMURA, M., NAKA, T., OJIMA, T., UEDA, K., HAYATA, K., NAKAMURA, Y. & YAMAUE, H. (2011) Tumor-infiltrating CD4+ Th17 cells produce IL-17 in tumor microenvironment and promote tumor progression in human gastric cancer. *Oncology Reports,* 25, 1271-7.

INATSU, A., KOGISO, M., JESCHKE, M. G., ASAI, A., KOBAYASHI, M., HERNDON, D. N. & SUZUKI, F. (2011) Lack of Th17 cell generation in patients with severe burn injuries. *Journal of Immunology,* 187, 2155-61.

INTLEKOFER, A. M., BANERJEE, A., TAKEMOTO, N., GORDON, S. M., DEJONG, C. S., SHIN, H., HUNTER, C. A., WHERRY, E. J., LINDSTEN, T. & REINER, S. L. (2008) Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science,* 321, 408-11.

ISHIGAME, H., KAKUTA, S., NAGAI, T., KADOKI, M., NAMBU, A., KOMIYAMA, Y., FUJIKADO, N., TANAHASHI, Y., AKITSU, A., KOTAKI, H., SUDO, K., NAKAE, S., SASAKAWA, C. & IWAKURA, Y. (2009) Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity,* 30, 108-19.

ISOMOTO, H., MUKAE, H., ISHIMOTO, H., DATE, Y., NISHI, Y., INOUE, K., WADA, A., HIRAYAMA, T., NAKAZATO, M. & KOHNO, S. (2004) Elevated Concentrations of [alpha]-Defensins in Gastric Juice of Patients with Helicobacter pylori Infection. *Am J Gastroenterol,* 99, 1916-1923.

ITO, Y., USUI, T., KOBAYASHI, S., IGUCHI-HASHIMOTO, M., ITO, H., YOSHITOMI, H., NAKAMURA, T., SHIMIZU, M., KAWABATA, D., YUKAWA, N., HASHIMOTO, M., SAKAGUCHI, N., SAKAGUCHI, S., YOSHIFUJI, H., NOJIMA, T., OHMURA, K., FUJII, T. & MIMORI, T. (2009) Gamma/delta T cells are the predominant source of interleukin-17 in affected joints in collagen-induced arthritis, but not in rheumatoid arthritis. *Arthritis & Rheumatism,* 60, 2294-303.

ITOH, T., WAKATSUKI, Y., YOSHIDA, M., USUI, T., MATSUNAGA, Y., KANEKO, S., CHIBA, T. & KITA, T. (1999) The vast majority of gastric T cells are polarized to produce T helper 1 type cytokines upon antigenic stimulation despite the absence of Helicobacter pylori infection.[see comment]. *Journal of Gastroenterology,* 34, 560-70.

ITZKOWITZ, S. H. & YIO, X. (2004) Inflammation and Cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* 287, G7-G17.

IVANOV, I. I., MCKENZIE, B. S., ZHOU, L., TADOKORO, C. E., LEPELLEY, A., LAFAILLE, J. J., CUA, D. J. & LITTMAN, D. R. (2006) The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell,* 126, 1121-33.

IWATA, M., HIRAKIYAMA, A., ESHIMA, Y., KAGECHIKA, H., KATO, C. & SONG, S.-Y. (2004) Retinoic Acid Imprints Gut-Homing Specificity on T cells. *Immunity,* 21, 527-538.

JACKSON, A. M., MULCAHY, L. A., PORTE, J., FRANKS, H. A., EL REFAEE, M., WANG, Q., SHAH, S., ZHU, X. & PATEL, P. M. Role of mitogen-activated protein kinase and PI3K pathways in the regulation of IL-12-family cytokines in dendritic cells and the generation of T H-responses. *European Cytokine Network,* 21, 319-28.

JACKSON, L., BRITTON, J., LEWIS, S. A., MCKEEVER, T. M., ATHERTON, J., FULLERTON, D. & FOGARTY, A. W. (2009) A population-based epidemiologic study of Helicobacter pylori infection and its association with systemic inflammation. *Helicobacter,* 14, 108-13.

JAFARZADEH, A., MIRZAEE, V., AHMAD-BEYGI, H., NEMATI, M. & REZAYATI, M. T. (2009) Association of the CagA status of Helicobacter pylori and serum levels of interleukin

(IL)-17 and IL-23 in duodenal ulcer patients. *Journal of Digestive Diseases,* 10, 107-12.

JAIN, P., LUO, Z.-Q. & BLANKE, S. R. (2011) Helicobacter pylori vacuolating cytotoxin A (VacA) engages the mitochondrial fission machinery to induce host cell death. *Proceedings of the National Academy of Sciences.*

JAKOB, B., BIRKHOLZ, S., SCHNEIDER, T., DUCHMANN, R., ZEITZ, M. & STALLMACH, A. (2001) Immune response to autologous and heterologous Helicobacter pylori antigens in humans. *Microscopy Research & Technique,* 53, 419-24.

JAKSZYN, P., BINGHAM, S., PERA, G., AGUDO, A., LUBEN, R., WELCH, A., BOEING, H., DEL GIUDICE, G., PALLI, D., SAIEVA, C., KROGH, V., SACERDOTE, C., TUMINO, R., PANICO, S., BERGLUND, G., SIMAN, H., HALLMANS, G., SANCHEZ, M. J., LARRANAGA, N., BARRICARTE, A., CHIRLAQUE, M. D., QUIROS, J. R., KEY, T. J., ALLEN, N., LUND, E., CARNEIRO, F., LINSEISEN, J., NAGEL, G., OVERVAD, K., TJONNELAND, A., OLSEN, A., BUENO-DE-MESQUITA, H. B., OCKE, M. O., PEETERS, P. H., NUMANS, M. E., CLAVEL-CHAPELON, F., TRICHOPOULOU, A., FENGER, C., STENLING, R., FERRARI, P., JENAB, M., NORAT, T., RIBOLI, E. & GONZALEZ, C. A. (2006) Endogenous versus exogenous exposure to N-nitroso compounds and gastric cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) study. *Carcinogenesis,* 27, 1497-501.

JANA, M., DASGUPTA, S., PAL, U. & PAHAN, K. (2009) IL-12 p40 homodimer, the so-called biologically inactive molecule, induces nitric oxide synthase in microglia via IL-12R beta 1. *GLIA,* 57, 1553-65.

JANA, M., DASGUPTA, S., SAHA, R. N., LIU, X. & PAHAN, K. (2003) Induction of tumor necrosis factor-alpha (TNF-alpha) by interleukin-12 p40 monomer and homodimer in microglia and macrophages. *Journal of Neurochemistry,* 86, 519-28.

JANA, M. & PAHAN, K. (2009) Induction of lymphotoxin-alpha by interleukin-12 p40 homodimer, the so-called biologically inactive molecule, but not IL-12 p70. *Immunology,* 127, 312-25.

JANEWAY, C. A., TRAVERS, P., WALPORT, M. & SHLOMCHIK, M. J. (2005) *Immunobiology: the immune system in health and disease.*

JANG, M. H., KWEON, M.-N., IWATANI, K., YAMAMOTO, M., TERAHARA, K., SASAKAWA, C., SUZUKI, T., NOCHI, T., YOKOTA, Y., RENNERT, P. D., HIROI, T., TAMAGAWA, H., IIJIMA, H., KUNISAWA, J., YUKI, Y. & KIYONO, H. (2004) Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proceedings of the National Academy of Sciences of the United States of America,* 101, 6110-5.

JANG, T. J. (2010) The number of Foxp3-positive regulatory T cells is increased in Helicobacter pylori gastritis and gastric cancer. *Pathology - Research and Practice,* 206, 34-38.

JEMAL, A., BRAY, F., CENTER, M. M., FERLAY, J., WARD, E. & FORMAN, D. (2011) Global cancer statistics. *CA: A Cancer Journal for Clinicians,* 61, 69-90.

JIANG, R., TAN, Z., DENG, L., CHEN, Y., XIA, Y., GAO, Y., WANG, X. & SUN, B. (2011) Interleukin-22 promotes human hepatocellular carcinoma by activation of STAT3. *Hepatology,* 54, 900-9.

JOHANSSON-LINDBOM, B., SVENSSON, M., PABST, O., PALMQVIST, C., MARQUEZ, G., FORSTER, R. & AGACE, W. W. (2005) Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *Journal of Experimental Medicine,* 202, 1063-73.

JUNG, S. W., SUGIMOTO, M., SHIOTA, S., GRAHAM, D. Y. & YAMAOKA, Y. (2012) The Intact dupA Cluster Is a More Reliable Helicobacter pylori Virulence Marker than dupA Alone. *Infection and Immunity,* 80, 381-387.

JURADO, J. O., PASQUINELLI, V., ALVAREZ, I. B., PENA, D., ROVETTA, A. I., TATEOSIAN, N. L., ROMEO, H. E., MUSELLA, R. M., PALMERO, D., CHULUYAN, H. E. & GARCIA, V. E. (2012) IL-17 and IFN-gamma expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease. *Journal of Leukocyte Biology,* 91, 991-1002.

KADOWAKI, N., HO, S., ANTONENKO, S., MALEFYT, R. W., KASTELEIN, R. A., BAZAN, F. & LIU, Y. J. (2001) Subsets of human dendritic cell precursors express different toll-like

receptors and respond to different microbial antigens. *Journal of Experimental Medicine,* 194, 863-9.

KAGAMI, S., RIZZO, H. L., LEE, J. J., KOGUCHI, Y. & BLAUVELT, A. (2010) Circulating Th17, Th22, and Th1 cells are increased in psoriasis. *Journal of Investigative Dermatology,* 130, 1373-83.

KANDULSKI, A., WEX, T., KUESTER, D., PEITZ, U., GEBERT, I., ROESSNER, A. & MALFERTHEINER, P. (2008) Naturally occurring regulatory T cells (CD4+, CD25high, FOXP3+) in the antrum and cardia are associated with higher H. pylori colonization and increased gene expression of TGF-beta1. *Helicobacter,* 13, 295-303.

KAO, C.-Y., CHEN, Y., THAI, P., WACHI, S., HUANG, F., KIM, C., HARPER, R. W. & WU, R. (2004) IL-17 Markedly Up-Regulates b-Defensin-2 Expression in Human Airway Epithelium via JAK and NF-kB Signaling Pathways. *The Journal of Immunology,* 173, 3482-3491.

KAO, C.-Y., HUANG, F., CHEN, Y., THAI, P., WACHI, S., KIM, C., TAM, L. & WU, R. (2005) Up-Regulation of CC Chemokine Ligand 20 Expression in Human Airway Epithelium by IL-17 through a JAK-Independent but MEK/NF-kB-Dependent Signaling Pathway. *The Journal of Immunology,* 175, 6676-6685.

KAO, J. Y., PIERZCHALA, A., RATHINAVELU, S., ZAVROS, Y., TESSIER, A. & MERCHANT, J. L. (2006a) Somatostatin inhibits dendritic cell responsiveness to Helicobacter pylori. *Regulatory Peptides,* 134, 23-9.

KAO, J. Y., RATHINAVELU, S., EATON, K. A., BAI, L., ZAVROS, Y., TAKAMI, M., PIERZCHALA, A. & MERCHANT, J. L. (2006b) Helicobacter pylori-secreted factors inhibit dendritic cell IL-12 secretion: a mechanism of ineffective host defense. *American Journal of Physiology - Gastrointestinal & Liver Physiology,* 291, G73-81.

KAO, J. Y., ZHANG, M., MILLER, M. J., MILLS, J. C., WANG, B., LIU, M., EATON, K. A., ZOU, W., BERNDT, B. E., COLE, T. S., TAKEUCHI, T., OWYANG, S. Y. & LUTHER, J. (2010) Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. *Gastroenterology,* 138, 1046-54.

KAPSENBERG, M. L. (2003) Dendritic-cell control of pathogen-driven T-cell polarization. *Nature Reviews. Immunology,* 3, 984-93.

KARANFILOV, C. I., LIU, B., FOX, C. C., LAKSHMANAN, R. R. & WHISLER, R. L. (1999) Age-related defects in Th1 and Th2 cytokine production by human T cells can be dissociated from altered frequencies of CD45RA+ and CD45RO+ T cell subsets. *Mechanisms of Ageing & Development,* 109, 97-112.

KARTTUNEN, R. (1991) Blood lymphocyte proliferation, cytokine secretion and appearance of T cells with activation surface markers in cultures with Helicobacter pylori. Comparison of the responses of subjects with and without antibodies to H. pylori. *Clinical & Experimental Immunology,* 83, 396-400.

KARTTUNEN, R., ANDERSSON, G., POIKONEN, K., KOSUNEN, T. U., KARTTUNEN, T., JUUTINEN, K. & NIEMELA, S. (1990) Helicobacter pylori induces lymphocyte activation in peripheral blood cultures. *Clinical & Experimental Immunology,* 82, 485-8.

KARTTUNEN, R., KARTTUNEN, T., EKRE, H. P. & MACDONALD, T. T. (1995) Interferon gamma and interleukin 4 secreting cells in the gastric antrum in Helicobacter pylori positive and negative gastritis. *Gut,* 36, 341-5.

KATO, S., FURUYAMA, N., OZAWA, K., OHNUMA, K. & IINUMA, K. (1999) Long-term follow-up study of serum immunoglobulin G and immunoglobulin A antibodies after Helicobacter pylori eradication. *Pediatrics,* 104, e22.

KATO, T., FURUMOTO, H., OGURA, T., ONISHI, Y., IRAHARA, M., YAMANO, S., KAMADA, M. & AONO, T. (2001) Expression of IL-17 mRNA in Ovarian Cancer. *Biochemical and Biophysical Research Communications,* 282, 735-738.

KAWAI, T. & AKIRA, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol,* 11, 373-384.

KAWAKUBO, M., ITO, Y., OKIMURA, Y., KOBAYASHI, M., SAKURA, K., KASAMA, S., FUKUDA, M. N., FUKUDA, M., KATSUYAMA, T. & NAKAYAMA, J. (2004) Natural antibiotic function of a human gastric mucin against Helicobacter pylori infection. *Science,* 305, 1003-6.

KAYHAN, B., ARASLI, M., EREN, H., AYDEMIR, S., KAYHAN, B., AKTAS, E. & TEKIN, I. (2008) Analysis of peripheral blood lymphocyte phenotypes and Th1/Th2 cytokines profile

in the systemic immune responses of Helicobacter pylori infected individuals. *Microbiology & Immunology,* 52, 531-8.

KEATES, S., KEATES, A. C., KATCHAR, K., PEEK, R. M., JR. & KELLY, C. P. (2007) Helicobacter pylori induces up-regulation of the epidermal growth factor receptor in AGS gastric epithelial cells.[see comment]. *Journal of Infectious Diseases,* 196, 95-103.

KEBIR, H., IFERGAN, I., ALVAREZ, J. I., BERNARD, M., POIRIER, J., ARBOUR, N., DUQUETTE, P. & PRAT, A. (2009) Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Annals of Neurology,* 66, 390-402.

KENEFECK, R. M. W. (2008) PhD thesis: Human CD4 T-cell Responses in Helicobacter pylori Infection and Disease. University of Nottingham.

KENEFECK, R. M. W., ATHERTON, J. C. & ROBINSON, K. (2007) Human Helicobacter pylori infection induces $CD4^{+}CD25^{hi}IL\text{-}10^{+}$ Regulatory T-cells in the peripheral blood. *Zoonoses and Public Health,* 54 (suppl. 1).

KENNA, T. J., DAVIDSON, S. I., DUAN, R., BRADBURY, L. A., MCFARLANE, J., SMITH, M., WEEDON, H., STREET, S., THOMAS, R., THOMAS, G. P. & BROWN, M. A. (2012) Enrichment of circulating interleukin-17-secreting interleukin-23 receptor-positive gamma/delta T cells in patients with active ankylosing spondylitis. *Arthritis & Rheumatism,* 64, 1420-9.

KESZEI, A. S. P., GOLDBOHM, R. A., SCHOUTEN, L. J., JAKSZYN, P. & VAN DEN BRANDT, P. A. (2013) Dietary N-nitroso compounds, endogenous nitrosation, and the risk of esophageal and gastric cancer subtypes in the Netherlands Cohort Study. *The American Journal of Clinical Nutrition,* 97, 135-146.

KHADER, S. A., GAFFEN, S. L. & KOLLS, J. K. (2009) Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal immunology,* 2, 403-11.

KHAMRI, W., WALKER, M. M., CLARK, P., ATHERTON, J. C., THURSZ, M. R., BAMFORD, K. B., LECHLER, R. I. & LOMBARDI, G. (2010) Helicobacter pylori stimulates dendritic cells to induce interleukin-17 expression from CD4+ T lymphocytes. *Infection & Immunity,* 78, 845-53.

KHATTRI, R., COX, T., YASAYKO, S.-A. & RAMSDELL, F. (2003) An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nature Immunology,* 4, 337-342.

KIDO, M., TANAKA, J., AOKI, N., IWAMOTO, S., NISHIURA, H., CHIBA, T. & WATANABE, N. (2010) Helicobacter pylori promotes the production of thymic stromal lymphopoietin by gastric epithelial cells and induces dendritic cell-mediated inflammatory Th2 responses. *Infection & Immunity,* 78, 108-14.

KIM, D. J., PARK, K.-S., KIM, J.-H., YANG, S.-H., YOON, J. Y., HAN, B.-G., KIM, H. S., LEE, S. J., JANG, J. Y., KIM, K. H., KIM, M. J., SONG, J.-S., KIM, H.-J., PARK, C.-M., LEE, S.-K., LEE, B. I. & SUH, S. W. Helicobacter pylori proinflammatory protein up-regulates NF-kappaB as a cell-translocating Ser/Thr kinase. *Proceedings of the National Academy of Sciences of the United States of America,* 107, 21418-23.

KIM, J., CHO, Y. A., CHOI, I. J., LEE, Y.-S., KIM, S.-Y., SHIN, A., CHO, S.-J., KOOK, M.-C., NAM, J. H., RYU, K. W., LEE, J. H. & KIM, Y.-W. (2012) Effects of Interleukin-10 Polymorphisms, Helicobacter pylori Infection, and Smoking on the Risk of Noncardia Gastric Cancer. *PLoS ONE,* 7, e29643.

KIM, J. J., TAO, H., CARLONI, E., LEUNG, W. K., GRAHAM, D. Y. & SEPULVEDA, A. R. (2002) Helicobacter pylori impairs DNA mismatch repair in gastric epithelial cells. *Gastroenterology,* 123, 542-53.

KIMANG'A, A., REVATHI, G., KARIUKI, S., SAYED, S., DEVANI, S., VIVIENNE, M., KUESTER, D., MONKEMULLER, K., MALFERTHEINER, P. & WEX, T. IL-17A and IL-17F gene expression is strongly induced in the mucosa of H. pylori-infected subjects from Kenya and Germany. *Scandinavian Journal of Immunology,* 72, 522-8.

KINDLUND, B., SJÖLING, Å., HANSSON, M., EDEBO, A., HANSSON, L.-E., SJÖVALL, H., SVENNERHOLM, A.-M. & LUNDIN, B. S. (2009) FOXP3-expressing CD4+ T-cell Numbers Increase in Areas of Duodenal Gastric Metaplasia and are Associated to CD4+ T-cell Aggregates in the Duodenum of Helicobacter pylori-infected Duodenal Ulcer Patients. *Helicobacter,* 14, 192-201.

KIRIYA, K., WATANABE, N., NISHIO, A., OKAZAKI, K., KIDO, M., SAGA, K., TANAKA, J., AKAMATSU, T., OHASHI, S., ASADA, M., FUKUI, T. & CHIBA, T. (2007) Essential role of Peyer's patches in the development of Helicobacter-induced gastritis. *International Immunology,* 19, 435-46.

KLEINSCHEK, M. A., BONIFACE, K., SADEKOVA, S., GREIN, J., MURPHY, E. E., TURNER, S. P., RASKIN, L., DESAI, B., FAUBION, W. A., DE WAAL MALEFYT, R., PIERCE, R. H., MCCLANAHAN, T. & KASTELEIN, R. A. (2009) Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. *Journal of Experimental Medicine,* 206, 525-34.

KLOTZ, L., BURGDORF, S., DANI, I., SAIJO, K., FLOSSDORF, J., HUCKE, S., ALFERINK, J., NOWAK, N., BEYER, M., MAYER, G., LANGHANS, B., KLOCKGETHER, T., WAISMAN, A., EBERL, G., SCHULTZE, J., FAMULOK, M., KOLANUS, W., GLASS, C., KURTS, C. & KNOLLE, P. A. (2009) The nuclear receptor PPAR gamma selectively inhibits Th17 differentiation in a T cell-intrinsic fashion and suppresses CNS autoimmunity.[Erratum appears in J Exp Med. 2009 Dec 21;206(13):3159 Note: Novak, Natalija [corrected to Nowak, Nina]]. *Journal of Experimental Medicine,* 206, 2079-89.

KNELLER, R. W., YOU, W. C., CHANG, Y. S., LIU, W. D., ZHANG, L., ZHAO, L., XU, G. W., FRAUMENI, J. F., JR. & BLOT, W. J. (1992) Cigarette smoking and other risk factors for progression of precancerous stomach lesions. *Journal of the National Cancer Institute,* 84, 1261-6.

KOENEN, H. J. P. M., SMEETS, R. L., VINK, P. M., VAN RIJSSEN, E., BOOTS, A. M. H. & JOOSTEN, I. (2008) Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood,* 112, 2340-52.

KONDO, T., TAKATA, H., MATSUKI, F. & TAKIGUCHI, M. (2009) Cutting edge: Phenotypic characterization and differentiation of human CD8+ T cells producing IL-17. *Journal of Immunology,* 182, 1794-8.

KORN, T., BETTELLI, E., GAO, W., AWASTHI, A., JAGER, A., STROM, T. B., OUKKA, M. & KUCHROO, V. K. (2007) IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature,* 448, 484-7.

KOTAKE, S., UDAGAWA, N., TAKAHASHI, N., MATSUZAKI, K., ITOH, K., ISHIYAMA, S., SAITO, S., INOUE, K., KAMATANI, N., GILLESPIE, M. T., MARTIN, T. J. & SUDA, T. (1999) IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *Journal of Clinical Investigation,* 103, 1345-52.

KRANZER, K., ECKHARDT, A., AIGNER, M., KNOLL, G., DEML, L., SPETH, C., LEHN, N., REHLI, M. & SCHNEIDER-BRACHERT, W. (2004) Induction of maturation and cytokine release of human dendritic cells by Helicobacter pylori. *Infection & Immunity,* 72, 4416-23.

KRANZER, K., SOLLNER, L., AIGNER, M., LEHN, N., DEML, L., REHLI, M. & SCHNEIDER-BRACHERT, W. (2005) Impact of Helicobacter pylori virulence factors and compounds on activation and maturation of human dendritic cells. *Infection & Immunity,* 73, 4180-9.

KRUEGER, G. G., LANGLEY, R. G., LEONARDI, C., YEILDING, N., GUZZO, C., WANG, Y., DOOLEY, L. T., LEBWOHL, M. & GROUP, C. P. S. (2007) A human interleukin-12/23 monoclonal antibody for the treatment of psoriasis. *New England Journal of Medicine,* 356, 580-92.

KRYCZEK, I., BANERJEE, M., CHENG, P., VATAN, L., SZELIGA, W., WEI, S., HUANG, E., FINLAYSON, E., SIMEONE, D., WELLING, T. H., CHANG, A., COUKOS, G., LIU, R. & ZOU, W. (2009a) Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood,* 114, 1141-9.

KRYCZEK, I., WEI, S., SZELIGA, W., VATAN, L. & ZOU, W. (2009b) Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood,* 114, 357-9.

KRYCZEK, I., WEI, S., ZOU, L., ALTUWAIJRI, S., SZELIGA, W., KOLLS, J., CHANG, A. & ZOU, W. (2007) Cutting Edge: Th17 and Regulatory T Cell Dynamics and the Regulation by IL-2 in the Tumor Microenvironment. *The Journal of Immunology,* 178, 6730-6733.

KUANG, D.-M., PENG, C., ZHAO, Q., WU, Y., ZHU, L.-Y., WANG, J., YIN, X.-Y., LI, L. & ZHENG, L. (2010) Tumor-activated monocytes promote expansion of IL-17-producing CD8+ T cells in hepatocellular carcinoma patients. *Journal of Immunology,* 185, 1544-9.

KULLBERG, M. C., JANKOVIC, D., FENG, C. G., HUE, S., GORELICK, P. L., MCKENZIE, B. S., CUA, D. J., POWRIE, F., CHEEVER, A. W., MALOY, K. J. & SHER, A. (2006) IL-23 plays a key role in Helicobacter hepaticus–induced T cell–dependent colitis. *The Journal of Experimental Medicine,* 203, 2485-94.

KUMAR PACHATHUNDIKANDI, S., BRANDT, S., MADASSERY, J. & BACKERT, S. Induction of TLR-2 and TLR-5 expression by Helicobacter pylori switches cagPAI-dependent signalling leading to the secretion of IL-8 and TNF-alpha. *PLoS ONE [Electronic Resource],* 6, e19614.

KURAISHY, A., KARIN, M. & GRIVENNIKOV, S. I. (2011) Tumor promotion via injury- and death-induced inflammation. *Immunity,* 35, 467-77.

KUSUGAMI, K., ANDO, T., IMADA, A., INA, K., OHSUGA, M., SHIMIZU, T., SAKAI, T., KONAGAYA, T. & KANEKO, H. (1999) Mucosal macrophage inflammatory protein-1alpha activity in Helicobacter pylori infection. *Journal of Gastroenterology & Hepatology,* 14, 20-6.

KWOK, S.-K., CHO, M.-L., PARK, M.-K., OH, H.-J., PARK, J.-S., HER, Y.-M., LEE, S.-Y., YOUN, J., JU, J. H., PARK, K. S., KIM, S.-I., KIM, H.-Y. & PARK, S.-H. (2012) Interleukin-21 promotes osteoclastogenesis in humans with rheumatoid arthritis and in mice with collagen-induced arthritis. *Arthritis & Rheumatism,* 64, 740-751.

LADEIRAS-LOPES, R., PEREIRA, A. K., NOGUEIRA, A., PINHEIRO-TORRES, T., PINTO, I., SANTOS-PEREIRA, R. & LUNET, N. (2008) Smoking and gastric cancer: systematic review and meta-analysis of cohort studies. *Cancer Causes & Control,* 19, 689-701.

LANGRISH, C. L., CHEN, Y., BLUMENSCHEIN, W. M., MATTSON, J., BASHAM, B., SEDGWICK, J. D., MCCLANAHAN, T., KASTELEIN, R. A. & CUA, D. J. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *Journal of Experimental Medicine,* 201, 233-40.

LANIER, L. L., CHANG, C. & PHILLIPS, J. H. (1994) Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *The Journal of Immunology,* 153, 2417-28.

LATHROP, S. K., BLOOM, S. M., RAO, S. M., NUTSCH, K., LIO, C.-W., SANTACRUZ, N., PETERSON, D. A., STAPPENBECK, T. S. & HSIEH, C.-S. (2011) Peripheral education of the immune system by colonic commensal microbiota. *Nature,* 478, 250-4.

LAUREN, P. (1965) THE TWO HISTOLOGICAL MAIN TYPES OF GASTRIC CARCINOMA: DIFFUSE AND SO-CALLED INTESTINAL-TYPE CARCINOMA. AN ATTEMPT AT A HISTO-CLINICAL CLASSIFICATION. *Acta Pathologica et Microbiologica Scandinavica,* 64, 31-49.

LAURENCE, A. & O'SHEA, J. J. (2007) TH-17 differentiation: of mice and men. *Nature Immunology,* 8, 903-905.

LAURENCE, A., TATO, C. M., DAVIDSON, T. S., KANNO, Y., CHEN, Z., YAO, Z., BLANK, R. B., MEYLAN, F., SIEGEL, R., HENNIGHAUSEN, L., SHEVACH, E. M. & O'SHEA, J. J. (2007) Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity,* 26, 371-81.

LAZAREVIC, V., CHEN, X., SHIM, J.-H., HWANG, E.-S., JANG, E., BOLM, A. N., OUKKA, M., KUCHROO, V. K. & GLIMCHER, L. H. (2011) T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. *Nature Immunology,* 12, 96-104.

LEE, S. K. & JOSENHANS, C. (2005) Helicobacter pylori and the innate immune system. *Ijmm International Journal of Medical Microbiology,* 295, 325-34.

LEE, Y. K., TURNER, H., MAYNARD, C. L., OLIVER, J. R., CHEN, D., ELSON, C. O. & WEAVER, C. T. (2009) Late developmental plasticity in the T helper 17 lineage. *Immunity,* 30, 92-107.

LEHMANN, F. S., TERRACCIANO, L., CARENA, I., BAERISWYL, C., DREWE, J., TORNILLO, L., DE LIBERO, G. & BEGLINGER, C. (2002) In situ correlation of cytokine secretion and apoptosis in Helicobacter pylori-associated gastritis. *American Journal of Physiology - Gastrointestinal & Liver Physiology,* 283, G481-8.

LEONARDI, C., MATHESON, R., ZACHARIAE, C., CAMERON, G., LI, L., EDSON-HEREDIA, E., BRAUN, D. & BANERJEE, S. (2012) Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. *New England Journal of Medicine,* 366, 1190-9.

LEONARDI, C. L., KIMBALL, A. B., PAPP, K. A., YEILDING, N., GUZZO, C., WANG, Y., LI, S., DOOLEY, L. T., GORDON, K. B. & INVESTIGATORS, P. S. (2008) Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1).[see comment]. *Lancet,* 371, 1665-74.

LEVI, S., BEARDSHALL, K., HADDAD, G., PLAYFORD, R., GHOSH, P. & CALAM, J. (1989) Campylobacter pylori and duodenal ulcers: the gastrin link. *Lancet,* 1, 1167-8.

LEVINGS, M. K., GREGORI, S., TRESOLDI, E., CAZZANIGA, S., BONINI, C. & RONCAROLO, M. G. (2005) Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood,* 105, 1162-9.

LI, W., MINOHARA, M., SU, J. J., MATSUOKA, T., OSOEGAWA, M., ISHIZU, T. & KIRA, J.-I. (2007) Helicobacter pylori infection is a potential protective factor against conventional multiple sclerosis in the Japanese population. *Journal of Neuroimmunology,* 184, 227-231.

LI, Z. & LI, J. (2006) Local expressions of TGF-b1, TGF-b1RI, CTGF, and Smad-7 in Helicobacter pylori-associated gastritis. *Scandinavian Journal of Gastroenterology,* 41, 1007-12.

LIANG, S. C., TAN, X.-Y., LUXENBERG, D. P., KARIM, R., DUNUSSI-JOANNOPOULOS, K., COLLINS, M. & FOUSER, L. A. (2006) Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *Journal of Experimental Medicine,* 203, 2271-9.

LIM, H. W., LEE, J., HILLSAMER, P. & KIM, C. H. (2008) Human Th17 Cells Share Major Trafficking Receptors with Both Polarized Effector T Cells and FOXP3 Regulatory T Cells. *The Journal of Immunology,* 180, 122-29.

LIN, J., COOK, N. R., ALBERT, C., ZAHARRIS, E., GAZIANO, J. M., VAN DENBURGH, M., BURING, J. E. & MANSON, J. E. (2009) Vitamins C and E and Beta Carotene Supplementation and Cancer Risk: A Randomized Controlled Trial. *Journal of the National Cancer Institute,* 101, 14-23.

LINDÉN, S., SEMINO-MORA, C., LIU, H., RICK, J. & DUBOIS, A. (2010) Role of Mucin Lewis Status in Resistance to Helicobacter pylori Infection in Pediatric Patients. *Helicobacter,* 15, 251-258.

LINDGREN, A., PAVLOVIC, V., FLACH, C.-F., SJOLING, A. & LUNDIN, S. Interferon-gamma secretion is induced in IL-12 stimulated human NK cells by recognition of Helicobacter pylori or TLR2 ligands. *Innate immunity,* 17, 191-203.

LINDHOLM, C., QUIDING-JARBRINK, M., LONROTH, H., HAMLET, A. & SVENNERHOLM, A. M. (1998) Local cytokine response in Helicobacter pylori-infected subjects. *Infection & Immunity,* 66, 5964-71.

LING, P., GATELY, M. K., GUBLER, U., STERN, A. S., LIN, P., HOLLFELDER, K., SU, C., PAN, Y. C. & HAKIMI, J. (1995) Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *Journal of Immunology,* 154, 116-27.

LIU, C. & RUSSELL, R. M. (2008) Nutrition and gastric cancer risk: an update. *Nutrition Reviews,* 66, 237-49.

LIU, W., PUTNAM, A. L., XU-YU, Z., SZOT, G. L., LEE, M. R., ZHU, S., GOTTLIEB, P. A., KAPRANOV, P., GINGERAS, T. R., FAZEKAS DE ST GROTH, B., CLAYBERGER, C., SOPER, D. M., ZIEGLER, S. F. & BLUESTONE, J. A. (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells. *The Journal of Experimental Medicine,* 203, 1701-11.

LOCHHEAD, P. & EL-OMAR, E. M. (2007) Helicobacter pylori infection and gastric cancer. *Best Practice & Research in Clinical Gastroenterology,* 21, 281-97.

LOCHNER, M., PEDUTO, L., CHERRIER, M., SAWA, S., LANGA, F., VARONA, R., RIETHMACHER, D., SI-TAHAR, M., DI SANTO, J. P. & EBERL, G. R. (2008) In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ $ROR\hat{I}^3t$+ T cells. *The Journal of Experimental Medicine,* 205, 1381-1393.

LOCKHART, E., GREEN, A. M. & FLYNN, J. L. (2006) IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *Journal of Immunology,* 177, 4662-9.

LOHR, J., KNOECHEL, B., WANG, J. J., VILLARINO, A. V. & ABBAS, A. K. (2006) Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *Journal of Experimental Medicine,* 203, 2785-91.

LU, H., HSU, P.-I., GRAHAM, D. Y. & YAMAOKA, Y. (2005) Duodenal ulcer promoting gene of Helicobacter pylori. *Gastroenterology,* 128, 833-848.

LU, K. T., KANNO, Y., CANNONS, J. L., HANDON, R., BIBLE, P., ELKAHLOUN, A. G., ANDERSON, S. M., WEI, L., SUN, H., O'SHEA, J. J. & SCHWARTZBERG, P. L. (2011) Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. *Immunity,* 35, 622-32.

LUNDGREN, A., STROMBERG, E., SJOLING, A., LINDHOLM, C., ENARSSON, K., EDEBO, A., JOHNSSON, E., SURI-PAYER, E., LARSSON, P., RUDIN, A., SVENNERHOLM, A.-M. & LUNDIN, B. S. (2005) Mucosal FOXP3-expressing $CD4^+$ $CD25^{high}$ regulatory T cells in *Helicobacter pylori*-infected patients. *Infection and Immunity,* 73, 523-31.

LUNDGREN, A., SURI-PAYER, E., ENARSSON, K., SVENNERHOLM, A.-M. & LUNDIN, B. S. (2003) *Helicobacter pylori*-specific CD4+ $CD25^{high}$ Regulatory T Cells Suppress Memory T-Cell Responses to *H. pylori* in Infected Individuals. *Infection and Immunity,* 71, 1755-1762.

LUNDIN, B. S., ENARSSON, K., KINDLUND, B., LUNDGREN, A., JOHNSSON, E., QUIDING-JARBRINK, M. & SVENNERHOLM, A.-M. (2007) The local and systemic T-cell response to Helicobacter pylori in gastric cancer patients is characterised by production of interleukin-10. *Clinical Immunology,* 125, 205-13.

LUZZA, F., PARRELLO, T., MONTELEONE, G., SEBKOVA, L., ROMANO, M., ZARRILLI, R., IMENEO, M. & PALLONE, F. (2000) Up-Regulation of IL-17 Is Associated with Bioactive IL-8 Expression in *Helicobacter pylori*-Infected Human Gastric Mucosa. *The Journal of Immunology,* 165, 5332-5337.

LUZZA, F., PARRELLO, T., SEBKOVA, L., PENSABENE, L., IMENEO, M., MANCUSO, M., LA VECCHIA, A. M., MONTELEONE, G., STRISCIUGLIO, P. & PALLONE, F. (2001) Expression of proinflammatory and Th1 but not Th2 cytokines is enhanced in gastric mucosa of Helicobacter pylori infected children. *Digestive & Liver Disease,* 33, 14-20.

MA, C. S., CHEW, G. Y. J., SIMPSON, N., PRIYADARSHI, A., WONG, M., GRIMBACHER, B., FULCHER, D. A., TANGYE, S. G. & COOK, M. C. (2008) Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *Journal of Experimental Medicine,* 205, 1551-7.

MADDUR, M. S., VANI, J., HEGDE, P., LACROIX-DESMAZES, S., KAVERI, S. V. & BAYRY, J. (2011) Inhibition of differentiation, amplification, and function of human TH17 cells by intravenous immunoglobulin. *Journal of Allergy & Clinical Immunology,* 127, 823-30.e1-7.

MAEKITA, T., NAKAZAWA, K., MIHARA, M., NAKAJIMA, T., YANAOKA, K., IGUCHI, M., ARII, K., KANEDA, A., TSUKAMOTO, T., TATEMATSU, M., TAMURA, G., SAITO, D., SUGIMURA, T., ICHINOSE, M. & USHIJIMA, T. (2006) High levels of aberrant DNA methylation in Helicobacter pylori-infected gastric mucosae and its possible association with gastric cancer risk. *Clinical Cancer Research,* 12, 989-95.

MALFERTHEINER, P., MEGRAUD, F., O'MORAIN, C., BAZZOLI, F., EL-OMAR, E., GRAHAM, D., HUNT, R., ROKKAS, T., VAKIL, N. & KUIPERS, E. J. (2007) Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut,* 56, 772-81.

MALFERTHEINER, P., MEGRAUD, F., O'MORAIN, C. A., ATHERTON, J., AXON, A. T. R., BAZZOLI, F., GENSINI, G. F., GISBERT, J. P., GRAHAM, D. Y., ROKKAS, T., EL-OMAR, E. M., KUIPERS, E. J. & EUROPEAN HELICOBACTER STUDY, G. (2012) Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut,* 61, 646-64.

MALFITANO, A. M., CAHILL, R., MITCHELL, P., FRANKEL, G., DOUGAN, G., BIFULCO, M., LOMBARDI, G., LECHLER, R. I. & BAMFORD, K. B. (2006) Helicobacter pylori has stimulatory effects on naive T cells. *Helicobacter,* 11, 21-30.

MANEL, N., UNUTMAZ, D. & LITTMAN, D. R. (2008) The differentiation of human T_H-17 cells requires transforming growth factor-β and induction of the nuclear receptor RORγt. *Nature Immunology,* 9, 641-9.

MANGAN, P. R., HARRINGTON, L. E., O'QUINN, D. B., HELMS, W. S., BULLARD, D. C., ELSON, C. O., HATTON, R. D., WAHL, S. M., SCHOEB, T. R. & WEAVER, C. T. (2006) Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature,* 441, 231-4.

MAROTTI, B., ROCCO, A., DE COLIBUS, P., COMPARE, D., DE NUCCI, G., STAIBANO, S., TATANGELO, F., ROMANO, M. & NARDONE, G. (2008) Interleukin-13 mucosal production in Helicobacter pylori-related gastric diseases. *Digestive & Liver Disease,* 40, 240-7.

MARSHALL, B. J. & WARREN, J. R. (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet,* 1, 1311-5.

MARTIN-OROZCO, N., MURANSKI, P., CHUNG, Y., YANG, X. O., YAMAZAKI, T., LU, S., HWU, P., RESTIFO, N. P., OVERWIJK, W. W. & DONG, C. (2009) T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity,* 31, 787-98.

MARTIN, B., HIROTA, K., CUA, D. J., STOCKINGER, B. & VELDHOEN, M. (2009) Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity,* 31, 321-30.

MASSAGUE, J. (1990) The Transforming Growth Factor-beta Family. *Annual Review of Cell Biology,* 6, 597-641.

MATSUMOTO, M., YOKOYAMA, H., SUZUKI, H., SHIRAISHI-YOKOYAMA, H. & HIBI, T. (2005a) Retinoic acid formation from retinol in the human gastric mucosa: role of class IV alcohol dehydrogenase and its relevance to morphological changes. *American Journal Physiology Gastrointestinal and Liver Physiology,* 289, 429-33.

MATSUMOTO, Y., BLANCHARD, T. G., DRAKES, M. L., BASU, M., REDLINE, R. W., LEVINE, A. D. & CZINN, S. J. (2005b) Eradication of Helicobacter pylori and Resolution of Gastritis in the Gastric Mucosa of IL-10-Deficient Mice. *Helicobacter,* 10, 407-415.

MATSUMOTO, Y., MARUSAWA, H., KINOSHITA, K., ENDO, Y., KOU, T., MORISAWA, T., AZUMA, T., OKAZAKI, I.-M., HONJO, T. & CHIBA, T. (2007) Helicobacter pylori infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. *Nature Medicine,* 13, 470-6.

MAZMANIAN, S. K., ROUND, J. L. & KASPER, D. L. (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature,* 453, 620-5.

MCGEACHY, M. J., BAK-JENSEN, K. S., CHEN, Y., TATO, C. M., BLUMENSCHEIN, W., MCCLANAHAN, T. & CUA, D. J. (2007) TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. *Nat Immunol,* 8, 1390-1397.

MCGEACHY, M. J., CHEN, Y., TATO, C. M., LAURENCE, A., JOYCE-SHAIKH, B., BLUMENSCHEIN, W. M., MCCLANAHAN, T. K., O'SHEA, J. J. & CUA, D. J. (2009) The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol,* 10, 314-324.

MEIER, S., STARK, R., FRENTSCH, M. & THIEL, A. (2008) The influence of different stimulation conditions on the assessment of antigen-induced CD154 expression on CD4+ T cells. *Cytometry Part A: The Journal of the International Society for Analytical Cytology,* 73, 1035-42.

MEYER, F., RAMANUJAM, K. S., GOBERT, A. P., JAMES, S. P. & WILSON, K. T. (2003) Cutting Edge: Cyclooxygenase-2 Activation Suppresses Th1 Polarization in Response to Helicobacter pylori. *The Journal of Immunology,* 171, 3913-17.

MIEHLKE, S., YU, J., SCHUPPLER, M., FRINGS, C., KIRSCH, C., NEGRASZUS, N., MORGNER, A., STOLTE, M., EHNINGER, G. & BAYERDORFFER, E. (2001) Helicobacter pylori vacA, iceA, and cagA status and pattern of gastritis in patients with malignant and benign gastroduodenal disease. *Am J Gastroenterol,* 96, 1008-1013.

MILJKOVIC, D. & TRAJKOVIC, V. (2004) Inducible nitric oxide synthase activation by interleukin-17. *Cytokine & Growth Factor Reviews,* 15, 21-32.

MILNER, J. D., BRENCHLEY, J. M., LAURENCE, A., FREEMAN, A. F., HILL, B. J., ELIAS, K. M., KANNO, Y., SPALDING, C., ELLOUMI, H. Z., PAULSON, M. L., DAVI, J., HSU, A., ASHER, A. I., O'SHEA, J., HOLLAND, S. M., PAUL, W. E. & DOUEK, D. C. (2008) Impaired TH17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature,* 452, 773-777.

MIRE-SLUIS, A. R., DAS, R. G. & PADILLA, A. (1998) WHO cytokine standardization: facilitating the development of cytokines in research, diagnosis and as therapeutic agents. *Journal of Immunological Methods,* 216, 103-16.

MITCHELL, P., GERMAIN, C., FIORI, P. L., KHAMRI, W., FOSTER, G. R., GHOSH, S., LECHLER, R. I., BAMFORD, K. B. & LOMBARDI, G. (2007) Chronic Exposure to Helicobacter pylori Impairs Dendritic Cell Function and Inhibits Th1 Development. *Infection and Immunity,* 75, 810-19.

MITCHELL, P. J., AFZALI, B., FAZEKASOVA, H., CHEN, D., ALI, N., POWELL, N., LORD, G. M., LECHLER, R. I. & LOMBARDI, G. (2012) Helicobacter pylori induces in-vivo expansion of human regulatory T cells through stimulating interleukin-1β production by dendritic cells. *Clinical & Experimental Immunology,* 170, 300-309.

MIYARA, M., YOSHIOKA, Y., KITOH, A., SHIMA, T., WING, K., NIWA, A., PARIZOT, C., TAFLIN, C. C., HEIKE, T., VALEYRE, D., MATHIAN, A., NAKAHATA, T., YAMAGUCHI, T., NOMURA, T., ONO, M., AMOURA, Z., GOROCHOV, G. & SAKAGUCHI, S. (2009) Functional Delineation and Differentiation Dynamics of Human CD4+ T Cells Expressing the FoxP3 Transcription Factor. *Immunity,* 30, 899-911.

MIZUKAMI, Y., KONO, K., KAWAGUCHI, Y., AKAIKE, H., KAMIMURA, K., SUGAI, H. & FUJII, H. (2008) Localisation pattern of Foxp3+ regulatory T cells is associated with clinical behaviour in gastric cancer. *British Journal of Cancer,* 98, 48-53.

MIZUNO, T., ANDO, T., NOBATA, K., TSUZUKI, T., MAEDA, O., WATANABE, O., MINAMI, M., INA, K., KUSUGAMI, K., PEEK, R. M. & GOTO, H. (2005) Interleukin-17 levels in Helicobacter pylori-infected gastric mucosa and pathologic sequelae of colonization. *World Journal of Gastroenterology,* 11, 6305-11.

MJOSBERG, J. M., TRIFARI, S., CRELLIN, N. K., PETERS, C. P., VAN DRUNEN, C. M., PIET, B., FOKKENS, W. J., CUPEDO, T. & SPITS, H. (2011) Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol,* 12, 1055-1062.

MOISAN, J., GRENNINGLOH, R., BETTELLI, E., OUKKA, M. & HO, I. C. (2007) Ets-1 is a negative regulator of Th17 differentiation. *Journal of Experimental Medicine,* 204, 2825-35.

MOLESWORTH-KENYON, S. J., YIN, R., OAKES, J. E. & LAUSCH, R. N. (2008) IL-17 receptor signaling influences virus-induced corneal inflammation. *Journal of Leukocyte Biology,* 83, 401-8.

MOLINARI, M., SALIO, M., GALLI, C., NORAIS, N., RAPPUOLI, R., LANZAVECCHIA, A. & MONTECUCCO, C. (1998) Selective Inhibition of Ii-dependent Antigen Presentation by Helicobacter pylori Toxin VacA. *Journal of Experimental Medicine,* 187, 135-40.

MONTELEONE, G., CARUSO, R., FINA, D., PELUSO, I., GIOIA, V., STOLFI, C., FANTINI, M. C., CAPRIOLI, F., TERSIGNI, R., ALESSANDRONI, L., MACDONALD, T. T. & PALLONE, F. (2006) Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. *Gut,* 55, 1774-80.

MONTELEONE, G., MONTELEONE, I., FINA, D., VAVASSORI, P., DEL VECCHIO BLANCO, G., CARUSO, R., TERSIGNI, R., ALESSANDRONI, L., BIANCONE, L., NACCARI, G. C., MACDONALD, T. T. & PALLONE, F. (2005) Interleukin-21 enhances T-helper cell type I signaling and interferon-gamma production in Crohn's disease. *Gastroenterology,* 128, 687-94.

MONTELEONE, G., PALLONE, F. & MACDONALD, T. T. (2009) Interleukin-21 as a new therapeutic target for immune-mediated diseases. *Trends in Pharmacological Sciences,* 30, 441-447.

MORA, J. R., BONO, M. R., MANJUNATH, N., WENINGER, W., CAVANAGH, L. L., ROSEMBLATT, M. & VON ANDRIAN, U. H. (2003) Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature,* 424, 88-93.

MORA, J. R., IWATA, M., EKSTEEN, B., SONG, S.-Y., JUNT, T., SENMAN, B., OTIPOBY, K. L., YOKOTA, A., TAKEUCHI, H., RICCIARDI-CASTAGNOLI, P., RAJEWSKY, K., ADAMS, D. H. & VON ANDRIAN, U. H. (2006) Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science,* 314, 1157-60.

MORGAN, J. P., ROBINS, R. A., DUA, H. S. & TIGHE, P. J. *S antigen specific effector T cell activation detected by cytokine flow cytometry. [Letter],* British Journal of Ophthalmology May 2002;86(5):517-520.

MOSMANN, T. R. & COFFMAN, R. L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology,* 7, 145-73.

MOSMANN, T. R., LI, L. & SAD, S. (1997) Functions of CD8 T-cell subsets secreting different cytokine patterns. *Seminars in Immunology,* 9, 87-92.

MOSS, S. F., LEGON, S., DAVIES, J. & CALAM, J. (1994) Cytokine gene expression in Helicobacter pylori associated antral gastritis. *Gut,* 35, 1567-70.

MUCIDA, D., PARK, Y., KIM, G., TUROVSKAYA, O., SCOTT, I., KRONENBERG, M. & CHEROUTRE, H. (2007) Reciprocal TH17 and RegulatoryT Cell Differentiation Mediated by Retinoic Acid. *Science,* 317, 256-60.

MUELLER, A., SAYI, A. & HITZLER, I. (2009) Protective and pathogenic functions of T-cells are inseparable during the Helicobacter-host interaction. *Discovery Medicine,* 8, 68-73.

MURANSKI, P., BONI, A., ANTONY, P. A., CASSARD, L., IRVINE, K. R., KAISER, A., PAULOS, C. M., PALMER, D. C., TOULOUKIAN, C. E., PTAK, K., GATTINONI, L., WRZESINSKI, C., HINRICHS, C. S., KERSTANN, K. W., FEIGENBAUM, L., CHAN, C.-C. & RESTIFO, N. P. (2008) Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood,* 112, 362-73.

MURPHY, C. A., LANGRISH, C. L., CHEN, Y., BLUMENSCHEIN, W., MCCLANAHAN, T., KASTELEIN, R. A., SEDGWICK, J. D. & CUA, D. J. (2003) Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *Journal of Experimental Medicine,* 198, 1951-7.

NAGAI, S., MIMURO, H., YAMADA, T., BABA, Y., MORO, K., NOCHI, T., KIYONO, H., SUZUKI, T., SASAKAWA, C. & KOYASU, S. (2007) Role of Peyer's patches in the induction of Helicobacter pylori-induced gastritis. *Proceedings of the National Academy of Sciences of the United States of America,* 104, 8971-6.

NAKAJIMA, T., YAMASHITA, S., MAEKITA, T., NIWA, T., NAKAZAWA, K. & USHIJIMA, T. (2009) The presence of a methylation fingerprint of Helicobacter pylori infection in human gastric mucosae. *International Journal of Cancer,* 124, 905-10.

NAKAYAMADA, S., TAKAHASHI, H., KANNO, Y. & O'SHEA, J. J. Helper T cell diversity and plasticity. *Current Opinion in Immunology,* 24, 297-302.

NAPOLITANI, G., RINALDI, A., BERTONI, F., SALLUSTO, F. & LANZAVECCHIA, A. (2005) Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nature Immunology,* 6, 769-76.

NAVAGLIA, F., BASSO, D., ZAMBON, C. F., PONZANO, E., CAENAZZO, L., GALLO, N., FALDA, A., BELLUCO, C., FOGAR, P., GRECO, E., DI MARIO, F., RUGGE, M. & PLEBANI, M. (2005) Interleukin 12 gene polymorphisms enhance gastric cancer risk in H pylori infected individuals. *Journal of Medical Genetics,* 42, 503-10.

NAYLOR, K., LI, G., VALLEJO, A. N., LEE, W.-W., KOETZ, K., BRYL, E., WITKOWSKI, J., FULBRIGHT, J., WEYAND, C. M. & GORONZY, J. J. (2005) The influence of age on T cell generation and TCR diversity. *Journal of Immunology,* 174, 7446-52.

NECCHI, V., MANCA, R., RICCI, V. & SOLCIA, E. (2009) Evidence for transepithelial dendritic cells in human H. pylori active gastritis. *Helicobacter,* 14, 208-22.

NETEA, M. G., NOLD-PETRY, C. A., NOLD, M. F., JOOSTEN, L. A. B., OPITZ, B., VAN DER MEER, J. H. M., VAN DE VEERDONK, F. L., FERWERDA, G., HEINHUIS, B., DEVESA, I., FUNK, C. J., MASON, R. J., KULLBERG, B. J., RUBARTELLI, A., VAN DER MEER, J. W. M. & DINARELLO, C. A. (2009) Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood,* 113, 2324-35.

NIEDBALA, W., WEI, X.-Q., CAI, B., HUEBER, A. J., LEUNG, B. P., MCINNES, I. B. & LIEW, F. Y. (2007) IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *European Journal of Immunology,* 37, 3021-3029.

NIESS, J. H., BRAND, S., GU, X., LANDSMAN, L., JUNG, S., MCCORMICK, B. A., VYAS, J. M., BOES, M., PLOEGH, H. L., FOX, J. G., LITTMAN, D. R. & REINECKER, H.-C. (2005) CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science,* 307, 254-8.

NILSSON, I., KORNILOVS'KA, I., LINDGREN, S., LJUNGH, Ã. S. & WADSTRÃM, T. (2003) Increased prevalence of seropositivity for non-gastric Helicobacter species in patients with autoimmune liver disease. *Journal of Medical Microbiology,* 52, 949-953.

NISTALA, K., ADAMS, S., CAMBROOK, H., URSU, S., OLIVITO, B., DE JAGER, W., EVANS, J. G., CIMAZ, R., BAJAJ-ELLIOTT, M. & WEDDERBURN, L. R. (2010) Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proceedings of the National Academy of Sciences of the United States of America,* 107, 14751-6.

NOBELPRIZE.ORG (2005) The Nobel Prize in Physiology or Medicine 2005 Barry J. Marshall, J. Robin Warren. *http://www.nobelprize.org/nobel_prizes/medicine/laureates/2005/. Accessed 22/2/13.*

NOVATCHKOVA, M., LEIBBRANDT, A., WERZOWA, J., NEUBUSER, A. & EISENHABER, F. (2003) The STIR-domain superfamily in signal transduction, development and immunity. *Trends in Biochemical Sciences,* 28, 226-9.

NUMASAKI, M., FUKUSHI, J.-I., ONO, M., NARULA, S. K., ZAVODNY, P. J., KUDO, T., ROBBINS, P. D., TAHARA, H. & LOTZE, M. T. (2003) Interleukin-17 promotes angiogenesis and tumor growth. *Blood,* 101, 2620-2627.

NURGALIEVA, Z. Z., CONNER, M. E., OPEKUN, A. R., ZHENG, C. Q., ELLIOTT, S. N., ERNST, P. B., OSATO, M., ESTES, M. K. & GRAHAM, D. Y. (2005) B-cell and T-cell immune responses to experimental Helicobacter pylori infection in humans. *Infection & Immunity,* 73, 2999-3006.

NURIEVA, R., YANG, X. O., MARTINEZ, G., ZHANG, Y., PANOPOULOS, A. D., MA, L., SCHLUNS, K., TIAN, Q., WATOWICH, S. S., JETTEN, A. M. & DONG, C. (2007) Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature,* 448, 480-3.

NYIRENDA, M. H., SANVITO, L., DARLINGTON, P. J., O'BRIEN, K., ZHANG, G.-X., CONSTANTINESCU, C. S., BAR-OR, A. & GRAN, B. (2011) TLR2 stimulation drives human naive and effector regulatory T cells into a Th17-like phenotype with reduced suppressive function. *Journal of Immunology,* 187, 2278-90.

O'CONNOR, H. J. (1999) Review article: Helicobacter pylori and gastro-oesophageal reflux disease-clinical implications and management. *Alimentary Pharmacology & Therapeutics,* 13, 117-27.

O'KEEFFE, J., GATELY, C. M., O'DONOGHUE, Y., ZULQUERNAIN, S. A., STEVENS, F. M. & MORAN, A. P. (2008) Natural killer cell receptor T-lymphocytes in normal and Helicobacter pylori-infected human gastric mucosa. *Helicobacter,* 13, 500-5.

O'MAHONY, D. S., PHAM, U., IYER, R., HAWN, T. R. & LILES, W. C. (2008) Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages. *International Journal of Medical Sciences,* 5, 1-8.

OBONYO, M., GUINEY, D. G., HARWOOD, J., FIERER, J. & COLE, S. P. (2002) Role of gamma interferon in Helicobacter pylori induction of inflammatory mediators during murine infection. *Infection & Immunity,* 70, 3295-9.

OBONYO, M., RICKMAN, B. & GUINEY, D. G. (2011) Effects of myeloid differentiation primary response gene 88 (MyD88) activation on Helicobacter infection in vivo and induction of a Th17 response. *Helicobacter,* 16, 398-404.

OBST, B., WAGNER, S., SEWING, K. F. & BEIL, W. (2000) Helicobacter pylori causes DNA damage in gastric epithelial cells. *Carcinogenesis,* 21, 1111-5.

ODENBREIT, S., LINDER, S., GEBERT-VOGL, B., RIEDER, G., MORAN, A. P. & HAAS, R. (2006) Interleukin-6 induction by Helicobacter pylori in human macrophages is dependent on phagocytosis. *Helicobacter,* 11, 196-207.

OERTLI, M., NOBEN, M., ENGLER, D. B., SEMPER, R. P., REUTER, S., MAXEINER, J., GERHARD, M., TAUBE, C. & MULLER, A. (2013) Helicobacter pylori g-glutamyl transpeptidase and vacuolating cytotoxin promote gastric persistence and immune tolerance. *Proceedings of the National Academy of Sciences,* 110, 3047-3052.

OERTLI, M., SUNDQUIST, M., HITZLER, I., ENGLER, D. B., ARNOLD, I. C., REUTER, S., MAXEINER, J., HANSSON, M., TAUBE, C., QUIDING-JARBRINK, M. & MULLER, A. (2012) DC-

derived IL-18 drives Treg differentiation, murine Helicobacter pylori-specific immune tolerance, and asthma protection. *Journal of Clinical Investigation,* 122, 1082-96.

OKAMURA, H., TSUTSUI, H., KOMATSU, T., YUTSUDO, M., HAKURA, A., TANIMOTO, T., TORIGOE, K., OKURA, T., NUKADA, Y., HATTORI, K., AKITA, K., NAMBA, M., TANABE, F., KONISHI, K., FUKUDA, S. & KURIMOTO, M. (1995) Cloning of a new cytokine that induces IFN-[gamma] production by T cells. *Nature,* 378, 88-91.

OPPMANN, B., LESLEY, R., BLOM, B., TIMANS, J. C., XU, Y., HUNTE, B., VEGA, F., YU, N., WANG, J., SINGH, K., ZONIN, F., VAISBERG, E., CHURAKOVA, T., LIU, M., GORMAN, D., WAGNER, J., ZURAWSKI, S., LIU, Y., ABRAMS, J. S., MOORE, K. W., RENNICK, D., DE WAAL-MALEFYT, R., HANNUM, C., BAZAN, J. F. & KASTELEIN, R. A. (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity,* 13, 715-25.

ORTEGA, C., FERNANDEZ-A, S., CARRILLO, J. M., ROMERO, P., MOLINA, I. J., MORENO, J. C. & SANTAMARIA, M. (2009) IL-17-producing CD8+ T lymphocytes from psoriasis skin plaques are cytotoxic effector cells that secrete Th17-related cytokines. *Journal of Leukocyte Biology,* 86, 435-43.

OTANI, K., WATANABE, T., TANIGAWA, T., OKAZAKI, H., YAMAGAMI, H., WATANABE, K., TOMINAGA, K., FUJIWARA, Y., OSHITANI, N. & ARAKAWA, T. (2009) Anti-inflammatory effects of IL-17A on Helicobacter pylori-induced gastritis. *Biochemical and Biophysical Research Communications,* In Press, Uncorrected Proof.

OTT, J. J., ULLRICH, A., MASCARENHAS, M. & STEVENS, G. A. (2010) Global cancer incidence and mortality caused by behavior and infection. *Journal of Public Health,* 33, 223-233.

OTTE, J. M., NEUMANN, H. M., BRAND, S., SCHRADER, H., SCHMIDT, W. E. & SCHMITZ, F. (2009) Expression of beta-defensin 4 is increased in human gastritis. *European Journal of Clinical Investigation,* 39, 126-138.

OTTEMANN, K. M. & LOWENTHAL, A. C. (2002) Helicobacter pylori Uses Motility for Initial Colonization and To Attain Robust Infection. *Infection and Immunity,* 70, 1984-1990.

OUYANG, W., KOLLS, J. K. & ZHENG, Y. (2008) The Biological Functions of T Helper 17 Cell Effector Cytokines in Inflammation. *Immunity,* 28, 454-467.

OWEN, R. J. (1998) Helicobacter - species classification and identification. *British Medical Bulletin,* 54, 17-30.

PAHAN, K., SHEIKH, F. G., LIU, X., HILGER, S., MCKINNEY, M. & PETRO, T. M. (2001) Induction of nitric-oxide synthase and activation of NF-kappaB by interleukin-12 p40 in microglial cells. *Journal of Biological Chemistry,* 276, 7899-905.

PAPP, K. A., LEONARDI, C., MENTER, A., ORTONNE, J.-P., KRUEGER, J. G., KRICORIAN, G., ARAS, G., LI, J., RUSSELL, C. B., THOMPSON, E. H. Z. & BAUMGARTNER, S. (2012) Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis. *New England Journal of Medicine,* 366, 1181-9.

PAPPO, J., TORREY, D., CASTRIOTTA, L., SAVINAINEN, A., KABOK, Z. & IBRAGHIMOV, A. (1999) Helicobacter pylori infection in immunized mice lacking major histocompatibility complex class I and class II functions. *Infection & Immunity,* 67, 337-41.

PARK, H., LI, Z., YANG, X. O., CHANG, S. H., NURIEVA, R., WANG, Y.-H., WANG, Y., HOOD, L., ZHU, Z., TIAN, Q. & DONG, C. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature Immunology,* 6, 1133-41.

PARSONNET, J., VANDERSTEEN, D., GOATES, J., SIBLEY, R. K., PRITIKIN, J. & CHANG, Y. (1991) Helicobacter pylori infection in intestinal- and diffuse-type gastric adenocarcinomas.[Erratum appears in J Natl Cancer Inst 1991 Jun 19;83(12):881]. *Journal of the National Cancer Institute,* 83, 640-3.

PELCHEN-MATTHEWS, A., PARSONS, I. J. & MARSH, M. (1993) Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting. *Journal of Experimental Medicine,* 178, 1209-22.

PELETEIRO, B., LOPES, C., FIGUEIREDO, C. & LUNET, N. (2011) Salt intake and gastric cancer risk according to Helicobacter pylori infection, smoking, tumour site and histological type. *Br J Cancer,* 104, 198-207.

PELLICANÒ, A., IMENEO, M., LEONE, I., LARUSSA, T. & LUZZA, F. (2007) Enhanced Activation of Cyclooxygenase-2 Downregulates Th1 Signaling Pathway in *Helicobacter pylori*-infected Human Gastric Mucosa. *Helicobacter,* 12, 193-99.

PELLICANO, A., SEBKOVA, L., MONTELEONE, G., GUARNIERI, G., IMENEO, M., PALLONE, F. & LUZZA, F. (2007) Interleukin-12 Drives the Th1 Signaling Pathway in *Helicobacter pylori*-Infected Human Gastric Mucosa. *Infection and Immunity,* 75, 1738-44.

PELLICANO, R., FRANCESCHI, F., SARACCO, G., FAGOONEE, S., ROCCARINA, D. & GASBARRINI, A. (2009) Helicobacters and extragastric diseases. *Helicobacter,* 14 Suppl 1, 58-68.

PENE, J., CHEVALIER, S., PREISSER, L., VENEREAU, E., GUILLEUX, M.-H., GHANNAM, S., MOLES, J.-P., DANGER, Y., RAVON, E., LESAUX, S., YSSEL, H. & GASCAN, H. (2008) Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *Journal of Immunology,* 180, 7423-30.

PENG, M. Y., WANG, Z. H., YAO, C. Y., JIANG, L. N., JIN, Q. L., WANG, J. & LI, B. Q. (2008) Interleukin 17-producing gamma delta T cells increased in patients with active pulmonary tuberculosis. *Cellular & Molecular Immunology,* 5, 203-8.

PERFETTO, B., BUOMMINO, E., CANOZO, N., PAOLETTI, I., CORRADO, F., GRECO, R. & DONNARUMMA, G. (2004) Interferon-gamma cooperates with Helicobacter pylori to induce iNOS-related apoptosis in AGS gastric adenocarcinoma cells. *Research in Microbiology,* 155, 259-66.

PETERS, A., LEE, Y. & KUCHROO, V. K. (2011) The many faces of Th17 cells. *Current Opinion in Immunology,* 23, 702-706.

PFAFFL, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research,* 29, e45.

PICCOTTI, J. R., CHAN, S. Y., LI, K., EICHWALD, E. J. & BISHOP, D. K. (1997) Differential effects of IL-12 receptor blockade with IL-12 p40 homodimer on the induction of CD4+ and CD8+ IFN-gamma-producing cells. *Journal of Immunology,* 158, 643-8.

PICKERT, G., NEUFERT, C., LEPPKES, M., ZHENG, Y., WITTKOPF, N., WARNTJEN, M., LEHR, H.-A., HIRTH, S., WEIGMANN, B., WIRTZ, S., OUYANG, W., NEURATH, M. F. & BECKER, C. (2009) STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *Journal of Experimental Medicine,* 206, 1465-72.

POLK, D. B. & PEEK, R. M. (2010) Helicobacter pylori: gastric cancer and beyond. *Nat Rev Cancer,* 10, 403-414.

POT, C., APETOH, L., AWASTHI, A. & KUCHROO, V. K. (2011) Induction of regulatory Tr1 cells and inhibition of T(H)17 cells by IL-27. *Seminars in Immunology,* 23, 438-45.

PRENDERGAST, A., PRADO, J. G., KANG, Y.-H., CHEN, F., RIDDELL, L. A., LUZZI, G., GOULDER, P. & KLENERMAN, P. (2010) HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells. *AIDS,* 24, 491-502.

PRITCHARD, D. M. & PRZEMECK, S. M. C. (2004) Review article: how useful are the rodent animal models of gastric adenocarcinoma? *Alimentary Pharmacology and Therapeutics,* 19, 841-859.

PUEL, A., CYPOWYJ, S., BUSTAMANTE, J., WRIGHT, J. F., LIU, L., LIM, H. K., MIGAUD, M., ISRAEL, L., CHRABIEH, M., AUDRY, M., GUMBLETON, M., TOULON, A., BODEMER, C., EL-BAGHDADI, J., WHITTERS, M., PARADIS, T., BROOKS, J., COLLINS, M., WOLFMAN, N. M., AL-MUHSEN, S., GALICCHIO, M., ABEL, L., PICARD, C. & CASANOVA, J.-L. (2011) Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science,* 332, 65-8.

QIAN, Y., LIU, C., HARTUPEE, J., ALTUNTAS, C. Z., GULEN, M. F., JANE-WIT, D., XIAO, J., LU, Y., GILTIAY, N., LIU, J., KORDULA, T., ZHANG, Q.-W., VALLANCE, B., SWAIDANI, S., ARONICA, M., TUOHY, V. K., HAMILTON, T. & LI, X. (2007) The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. *Nature Immunology,* 8, 247-56.

QU, X.-H., HUANG, X.-L., XIONG, P., ZHU, C.-Y., HUANG, Y.-L., LU, L.-G., SUN, X., RONG, L., ZHONG, L., SUN, D.-Y., LIN, H., CAI, M.-C., CHEN, Z.-W., HU, B., WU, L.-M., JIANG, Y.-B. & YAN, W.-L. (2010) Does Helicobacter pylori infection play a role in iron deficiency anemia? A meta-analysis. *World Journal of Gastroenterology,* 16, 886-96.

QUIDING-JARBRINK, M., AHLSTEDT, I., LINDHOLM, C., JOHANSSON, E. L. & LONROTH, H. (2001a) Homing commitment of lymphocytes activated in the human gastric and intestinal mucosa. *Gut,* 49, 519-25.

QUIDING-JARBRINK, M., LUNDIN, B. S., LONROTH, H. & SVENNERHOLM, A. M. (2001b) CD4+ and CD8+ T cell responses in Helicobacter pylori-infected individuals. *Clinical & Experimental Immunology,* 123, 81-7.

QUIDING-JARBRINK, M., RAGHAVAN, S. & SUNDQUIST, M. (2010) Enhanced M1 macrophage polarization in human helicobacter pylori-associated atrophic gastritis and in vaccinated mice. *PLoS ONE [Electronic Resource],* 5, e15018.

QUINTANA, F. J., BASSO, A. S., IGLESIAS, A. H., KORN, T., FAREZ, M. F., BETTELLI, E., CACCAMO, M., OUKKA, M. & WEINER, H. L. (2008) Control of Treg and Th17 cell differentiation by the aryl hydrocarbon receptor. *Nature,* 453, 65-72.

RACHITSKAYA, A. V., HANSEN, A. M., HORAI, R., LI, Z., VILLASMIL, R., LUGER, D., NUSSENBLATT, R. B. & CASPI, R. R. (2008) Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. *Journal of Immunology,* 180, 5167-71.

RAD, R., BALLHORN, W., VOLAND, P., EISENACHER, K., MAGES, J., RAD, L., FERSTL, R., LANG, R., WAGNER, H., SCHMID, R. M., BAUER, S., PRINZ, C., KIRSCHNING, C. J. & KRUG, A. (2009) Extracellular and intracellular pattern recognition receptors cooperate in the recognition of Helicobacter pylori. *Gastroenterology,* 136, 2247-57.

RAD, R., BRENNER, L., BAUER, S., SCHWENDY, S., LAYLAND, L., DA COSTA, C. P., REINDL, W., DOSSUMBEKOVA, A., FRIEDRICH, M., SAUR, D., WAGNER, H., SCHMID, R. M. & PRINZ, C. (2006) CD25+/Foxp3+ T cells regulate gastric inflammation and Helicobacter pylori colonization in vivo. *Gastroenterology,* 131, 525-37.

RAD, R., BRENNER, L., KRUG, A., VOLAND, P., MAGES, J., LANG, R., SCHWENDY, S., REINDL, W., DOSSUMBEKOVA, A., BALLHORN, W., WAGNER, H., SCHMID, R. M., BAUER, S. & PRINZ, C. (2007) Toll-like receptor-dependent activation of antigen-presenting cells affects adaptive immunity to Helicobacter pylori. *Gastroenterology,* 133, 150-163.e3.

RAGHAVAN, S., FREDRIKSSON, M., SVENNERHOLM, A. M., HOLMGREN, J. & SURI-PAYER, E. (2003) Absence of CD4+CD25+ regulatory T cells is associated with a loss of regulation leading to increased pathology in Helicobacter pylori-infected mice. *Clinical & Experimental Immunology,* 132, 393-400.

RAMARAO, N., GRAY-OWEN, S. D. & MEYER, T. F. (2000) Helicobacter pylori induces but survives the extracellular release of oxygen radicals from professional phagocytes using its catalase activity. *Molecular Microbiology,* 38, 103-113.

RAMGOLAM, V. S., SHA, Y., JIN, J., ZHANG, X. & MARKOVIC-PLESE, S. (2009) IFN-beta Inhibits Human Th17 Cell Differentiation. *The Journal of Immunology,* 183, 5418-5427.

RANDOLPH, G. J., BEAULIEU, S., LEBECQUE, S., STEINMAN, R. M. & MULLER, W. A. (1998) Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science,* 282, 480-3.

RAO, V. P., POUTAHIDIS, T., GE, Z., NAMBIAR, P. R., BOUSSAHMAIN, C., WANG, Y. Y., HORWITZ, B. H., FOX, J. G. & ERDMAN, S. E. (2006) Innate Immune Inflammatory Response against Enteric Bacteria Helicobacter hepaticus Induces Mammary Adenocarcinoma in Mice. *Cancer Research,* 66, 7395-400.

REN, Z., PANG, G., LEE, R., BATEY, R., DUNKLEY, M., BORODY, T. & CLANCY, R. (2000) Circulating T-cell response to Helicobacter pylori infection in chronic gastritis. *Helicobacter,* 5, 135-41.

RES, P. C. M., PISKIN, G., DE BOER, O. J., VAN DER LOOS, C. M., TEELING, P., BOS, J. D. & TEUNISSEN, M. B. M. (2010) Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis. *PLoS ONE [Electronic Resource],* 5, e14108.

RESCIGNO, M., URBANO, M., VALZASINA, B., FRANCOLINI, M., ROTTA, G., BONASIO, R., GRANUCCI, F., KRAEHENBUHL, J. P. & RICCIARDI-CASTAGNOLI, P. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology,* 2, 361-7.

RHEAD, J. L., LETLEY, D. P., MOHAMMAD, M., HUSSEIN, N., MOHAGHEGHI, M. A., HOSSEINI, M. E. & ATHERTON, J. C. (2007) A New Helicobacter pylori Vacuolating Cytotoxin

Determinant, the Intermediate Region, Is Associated With Gastric Cancer. *Gastroenterology,* 133, 926-36.

RIBEIRO, S. M. F., POCZATEK, M., SCHULTZ-CHERRY, S., VILLAIN, M. & MURPHY-ULLRICH, J. E. (1999) The Activation Sequence of Thrombospondin-1 Interacts with the Latency-associated Peptide to Regulate Activation of Latent Transforming Growth Factor-Î². *Journal of Biological Chemistry,* 274, 13586-13593.

RICCI, C., HOLTON, J. & VAIRA, D. (2007) Diagnosis of Helicobacter pylori: invasive and non-invasive tests. *Best Practice & Research in Clinical Gastroenterology,* 21, 299-313.

RIMOLDI, M., CHIEPPA, M., SALUCCI, V., AVOGADRI, F., SONZOGNI, A., SAMPIETRO, G. M., NESPOLI, A., VIALE, G., ALLAVENA, P. & RESCIGNO, M. (2005) Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol,* 6, 507-514.

RIZWAN, M., ALVI, A. & AHMED, N. (2008) Novel protein antigen (JHP940) from the genomic plasticity region of Helicobacter pylori induces tumor necrosis factor alpha and interleukin-8 secretion by human macrophages. *Journal of Bacteriology,* 190, 1146-51.

ROBINSON, K., ARGENT, R. H. & ATHERTON, J. C. (2007) The inflammatory and immune response to Helicobacter pylori infection. *Best Practice & Research Clinical Gastroenterology,* 21, 237-59.

ROBINSON, K. & ATHERTON, J. (2009) *Book chapter: Helicobacter pylori* ASM Press, Washington D. C.

ROBINSON, K., KENEFECK, R., PIDGEON, E. L., SHAKIB, S., PATEL, S., POLSON, R. J., ZAITOUN, A. M. & ATHERTON, J. C. (2008) Helicobacter pylori-induced peptic ulcer disease is associated with inadequate regulatory T-cell responses. *Gut,* 57, 1375-85.

ROBINSON, K., NEAL, K. R., HOWARD, C., STOCKTON, J., ATKINSON, K., SCARTH, E., MORAN, J., ROBINS, A., TODD, I., KACZMARSKI, E., GRAY, S., MUSCAT, I., SLACK, R. & ALA'ALDEEN, D. A. A. (2002) Characterization of humoral and cellular immune responses elicited by meningococcal carriage. *Infection & Immunity,* 70, 1301-9.

ROCKALL, T. A., LOGAN, R. F., DEVLIN, H. B. & NORTHFIELD, T. C. (1995) Incidence of and mortality from acute upper gastrointestinal haemorrhage in the United Kingdom. Steering Committee and members of the National Audit of Acute Upper Gastrointestinal Haemorrhage. *BMJ,* 311, 222-6.

ROKKAS, T., PAPATHEODOROU, G., KARAMERIS, A., MAVROGEORGIS, A., KALOGEROPOULOS, N. & GIANNIKOS, N. (1995) Helicobacter pylori infection and gastric juice vitamin C levels. *Digestive Diseases and Sciences,* 40, 615-621.

ROKKAS, T., PISTIOLAS, D., SECHOPOULOS, P., ROBOTIS, I. & MARGANTINIS, G. (2007) Relationship between Helicobacter pylori infection and esophageal neoplasia: a meta-analysis. *Clinical Gastroenterology & Hepatology,* 5, 1413-7.

ROMANI, L., FALLARINO, F., DE LUCA, A., MONTAGNOLI, C., D'ANGELO, C., ZELANTE, T., VACCA, C., BISTONI, F., FIORETTI, M. C., GROHMANN, U., SEGAL, B. H. & PUCCETTI, P. (2008) Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature,* 451, 211-5.

ROMI, B., SOLDAINI, E., PANCOTTO, L., CASTELLINO, F., DEL GIUDICE, G. & SCHIAVETTI, F. (2011) Helicobacter pylori induces activation of human peripheral gammadelta+ T lymphocytes. *PLoS ONE [Electronic Resource],* 6, e19324.

ROOK, G. A. W. (2007) The hygiene hypothesis and the increasing prevalence of chronic inflammatory disorders. *Transactions of the Royal Society of Tropical Medicine & Hygiene,* 101, 1072-4.

ROSENSTIEL, P., HELLMIG, S., HAMPE, J., OTT, S., TILL, A., FISCHBACH, W., SAHLY, H., LUCIUS, R., FOLSCH, U. R., PHILPOTT, D. & SCHREIBER, S. (2006) Influence of polymorphisms in the NOD1/CARD4 and NOD2/CARD15 genes on the clinical outcome of Helicobacter pylori infection. *Cellular Microbiology,* 8, 1188-98.

ROSSI, R. L., ROSSETTI, G., WENANDY, L., CURTI, S., RIPAMONTI, A., BONNAL, R. J. P., BIROLO, R. S., MORO, M., CROSTI, M. C., GRUARIN, P., MAGLIE, S., MARABITA, F., MASCHERONI, D., PARENTE, V., COMELLI, M., TRABUCCHI, E., DE FRANCESCO, R., GEGINAT, J., ABRIGNANI, S. & PAGANI, M. (2011) Distinct microRNA signatures in

human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b. *Nature Immunology,* 12, 796-803.

ROTH, K. A., KAPADIA, S. B., MARTIN, S. M. & LORENZ, R. G. (1999) Cellular Immune Responses Are Essential for the Development of Helicobacter felis-Associated Gastric Pathology. *The Journal of Immunology,* 163, 1490-1497.

ROWELL, E. & WILSON, C. B. (2009) Programming perpetual T helper cell plasticity. *Immunity,* 30, 7-9.

RUSSELL, T. D., YAN, Q., FAN, G., KHALIFAH, A. P., BISHOP, D. K., BRODY, S. L. & WALTER, M. J. (2003) IL-12 p40 homodimer-dependent macrophage chemotaxis and respiratory viral inflammation are mediated through IL-12 receptor beta 1. *Journal of Immunology,* 171, 6866-74.

RUTZ, S., NOUBADE, R., EIDENSCHENK, C., OTA, N., ZENG, W., ZHENG, Y., HACKNEY, J., DING, J., SINGH, H. & OUYANG, W. (2011) Transcription factor c-Maf mediates the TGF-beta-dependent suppression of IL-22 production in T(H)17 cells. *Nature Immunology,* 12, 1238-45.

SA, S. M., VALDEZ, P. A., WU, J., JUNG, K., ZHONG, F., HALL, L., KASMAN, I., WINER, J., MODRUSAN, Z., DANILENKO, D. M. & OUYANG, W. (2007) The effects of IL-20 subfamily cytokines on reconstituted human epidermis suggest potential roles in cutaneous innate defense and pathogenic adaptive immunity in psoriasis.[Erratum appears in J Immunol. 2007 Jun 1;178(11):7487]. *Journal of Immunology,* 178, 2229-40.

SAKAGAMI, T., DIXON, M., O'ROURKE, J., HOWLETT, R., ALDERUCCIO, F., VELLA, J., SHIMOYAMA, T. & LEE, A. (1996) Atrophic gastric changes in both Helicobacter felis and Helicobacter pylori infected mice are host dependent and separate from antral gastritis. *Gut,* 39, 639-648.

SAKAGUCHI, S., SAKAGUCHI, N., ASANO, M., ITOH, M. & TODA, M. (1995) Immunologic Self-Tolerance Maintained by Activated T Cells Expressing IL-2 Receptor α-Chains (CD25) Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune Diseases. *Journal of Immunology,* 155, 1151-64.

SALLUSTO, F. & LANZAVECCHIA, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *Journal of Experimental Medicine,* 179, 1109-18.

SALLUSTO, F. & LANZAVECCHIA, A. (2002) The instructive role of dendritic cells on T-cell responses. *Arthritis Research,* 4 Suppl 3, S127-32.

SANDBORN, W. J., FEAGAN, B. G., FEDORAK, R. N., SCHERL, E., FLEISHER, M. R., KATZ, S., JOHANNS, J., BLANK, M., RUTGEERTS, P. & USTEKINUMAB CROHN'S DISEASE STUDY, G. (2008) A randomized trial of Ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease. *Gastroenterology,* 135, 1130-41.

SANDBORN, W. J., GASINK, C., GAO, L.-L., BLANK, M. A., JOHANNS, J., GUZZO, C., SANDS, B. E., HANAUER, S. B., TARGAN, S., RUTGEERTS, P., GHOSH, S., DE VILLIERS, W. J. S., PANACCIONE, R., GREENBERG, G., SCHREIBER, S., LICHTIGER, S., FEAGAN, B. G. & GROUP, C. S. (2012) Ustekinumab induction and maintenance therapy in refractory Crohn's disease. *New England Journal of Medicine,* 367, 1519-28.

SANTEGOETS, S. J. A. M., VAN DEN EERTWEGH, A. J. M., VAN DE LOOSDRECHT, A. A., SCHEPER, R. J. & DE GRUIJL, T. D. (2008) Human dendritic cell line models for DC differentiation and clinical DC vaccination studies. *Journal of Leukocyte Biology,* 84, 1364-73.

SATOH-TAKAYAMA, N., VOSSHENRICH, C. A. J., LESJEAN-POTTIER, S., SAWA, S., LOCHNER, M., RATTIS, F., MENTION, J.-J., THIAM, K., CERF-BENSUSSAN, N., MANDELBOIM, O., EBERL, G. & DI SANTO, J. P. (2008) Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity,* 29, 958-70.

SATOH, Y., OGAWARA, H., KAWAMURA, O., KUSANO, M. & MURAKAMI, H. Clinical Significance of Peripheral Blood T Lymphocyte Subsets in Helicobacter pylori-Infected Patients. *Gastroenterology research & practice,* 2012, 819842.

SAWAI, N., KITA, M., KODAMA, T., TANAHASHI, T., YAMAOKA, Y., TAGAWA, Y., IWAKURA, Y. & IMANISHI, J. (1999) Role of gamma interferon in Helicobacter pylori-induced gastric inflammatory responses in a mouse model. *Infection & Immunity,* 67, 279-85.

SAYI, A., KOHLER, E., HITZLER, I., ARNOLD, I., SCHWENDENER, R., REHRAUER, H. & MULLER, A. (2009) The CD4+ T cell-mediated IFN-gamma response to Helicobacter infection is essential for clearance and determines gastric cancer risk. *Journal of Immunology,* 182, 7085-101.

SAYI, A., KOHLER, E., TOLLER, I. M., FLAVELL, R. A., MULLER, W., ROERS, A. & MULLER, A. (2011) TLR-2-Activated B Cells Suppress Helicobacter-Induced Preneoplastic Gastric Immunopathology by Inducing T Regulatory-1 Cells. *The Journal of Immunology,* 186, 878-890.

SCHMAUSSER, B., ANDRULIS, M., ENDRICH, S., LEE, S. K., JOSENHANS, C., MULLER-HERMELINK, H. K. & ECK, M. (2004) Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection. *Clinical & Experimental Immunology,* 136, 521-6.

SEBKOVA, L., PELLICANO, A., MONTELEONE, G., GRAZIOLI, B., GUARNIERI, G., IMENEO, M., PALLONE, F. & LUZZA, F. (2004) Extracellular Signal-Related Protein Kinase Mediates Interleukin 17 (IL-17)-Induced IL-8 Secretion in *Helicobacter pylori*-Infected Human Gastric Epithelial Cells. *Infection and Immunity,* 72, 5019-5026.

SEDDIKI, N., SANTNER-NANAN, B., MARTINSON, J., ZAUNDERS, J., SASSON, S., LANDAY, A., SOLOMON, M., SELBY, W., ALEXANDER, S. I., NANAN, R., KELLEHER, A. & FAZEKAS DE ST GROTH, B. (2006) Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *Journal of Experimental Medicine,* 203, 1693-700.

SERAFINI, M., JAKSZYN, P., LUJÁN-BARROSO, L., AGUDO, A., BAS BUENO-DE-MESQUITA, H., VAN DUIJNHOVEN, F. J. B., JENAB, M., NAVARRO, C., PALLI, D., BOEING, H., WALLSTRÖM, P., REGNÉR, S., NUMANS, M. E., CARNEIRO, F., BOUTRON-RUAULT, M.-C., CLAVEL-CHAPELON, F., MOROIS, S., GRIONI, S., PANICO, S., TUMINO, R., SACERDOTE, C., RAMON QUIRÓS, J., MOLINA-MONTES, E., HUERTA CASTAÑO, J. M., BARRICARTE, A., AMIANO, P., KHAW, K.-T., WAREHAM, N., ALLEN, N. E., KEY, T. J., JEURNINK, S. M., PEETERS, P. H. M., BAMIA, C., VALANOU, E., TRICHOPOULOU, A., KAAKS, R., LUKANOVA, A., BERGMANN, M. M., LINDKVIST, B., STENLING, R., JOHANSSON, I., DAHM, C. C., OVERVAD, K., JENSEN, M., OLSEN, A., TJONNELAND, A., LUND, E., RINALDI, S., MICHAUD, D., MOUW, T., RIBOLI, E. & GONZÁLEZ, C. A. (2012) Dietary total antioxidant capacity and gastric cancer risk in the European prospective investigation into cancer and nutrition study. *International Journal of Cancer,* 131, E544-E554.

SERELLI-LEE, V., LING, K. L., HO, C., YEONG, L. H., LIM, G. K., HO, B. & WONG, S. B. J. (2012) Persistent Helicobacter pylori Specific Th17 Responses in Patients with Past *H. pylori* Infection Are Associated with Elevated Gastric Mucosal IL-1β. *PLoS ONE,* 7, e39199.

SEWALD, X., GEBERT-VOGL, B., PRASSL, S., BARWIG, I., WEISS, E., FABBRI, M., OSICKA, R., SCHIEMANN, M., BUSCH, D. H., SEMMRICH, M., HOLZMANN, B., SEBO, P. & HAAS, R. (2008) Integrin Subunit CD18 Is the T-Lymphocyte Receptor for the Helicobacter pylori Vacuolating Cytotoxin. *Cell Host & Microbe,* 3, 20-29.

SFANOS, K. S., BRUNO, T. C., MARIS, C. H., XU, L., THOBURN, C. J., DEMARZO, A. M., MEEKER, A. K., ISAACS, W. B. & DRAKE, C. G. (2008) Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing. *Clinical Cancer Research,* 14, 3254-61.

SHARMA, S. A., TUMMURU, M. K., BLASER, M. J. & KERR, L. D. (1998) Activation of IL-8 gene expression by Helicobacter pylori is regulated by transcription factor nuclear factor-kappa B in gastric epithelial cells. *Journal of Immunology,* 160, 2401-7.

SHARMA, S. A., TUMMURU, M. K., MILLER, G. G. & BLASER, M. J. (1995) Interleukin-8 response of gastric epithelial cell lines to Helicobacter pylori stimulation in vitro. *Infection & Immunity,* 63, 1681-87.

SHEN, H., GOODALL, J. C. & HILL GASTON, J. S. (2009) Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. *Arthritis & Rheumatism,* 60, 1647-56.

SHEN, Z., ZHOU, S., WANG, Y., LI, R.-L., ZHONG, C., LIANG, C. & SUN, Y. (2010) Higher intratumoral infiltrated Foxp3+ Treg numbers and Foxp3+/CD8+ ratio are associated with adverse prognosis in resectable gastric cancer. *Journal of Cancer Research and Clinical Oncology,* 136, 1585-1595.

SHI, G., COX, C. A., VISTICA, B. P., TAN, C., WAWROUSEK, E. F. & GERY, I. (2008) Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. *Journal of Immunology,* 181, 7205-13.

SHI, T., LIU, W.-Z., GAO, F., SHI, G.-Y. & XIAO, S.-D. (2005) Intranasal CpG-oligodeoxynucleotide is a potent adjuvant of vaccine against Helicobacter pylori, and T helper 1 type response and interferon-gamma correlate with the protection. *Helicobacter,* 10, 71-9.

SHI, Y., LIU, X.-F., ZHUANG, Y., ZHANG, J.-Y., LIU, T., YIN, Z., WU, C., MAO, X.-H., JIA, K.-R., WANG, F.-J., GUO, H., FLAVELL, R. A., ZHAO, Z., LIU, K.-Y., XIAO, B., GUO, Y., ZHANG, W.-J., ZHOU, W.-Y., GUO, G. & ZOU, Q.-M. (2010) Helicobacter pylori-induced Th17 responses modulate Th1 cell responses, benefit bacterial growth, and contribute to pathology in mice. *Journal of Immunology,* 184, 5121-9.

SHIBATA, K., YAMADA, H., HARA, H., KISHIHARA, K. & YOSHIKAI, Y. (2007) Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. *Journal of Immunology,* 178, 4466-72.

SHIBATA, T., TAHARA, T., HIRATA, I. & ARISAWA, T. (2009) Genetic polymorphism of interleukin-17A and -17F genes in gastric carcinogenesis. *Human Immunology,* 70, 547-551.

SHIMADA, M., ANDO, T., PEEK, R. M., WATANABE, O., ISHIGURO, K., MAEDA, O., ISHIKAWA, D., HASEGAWA, M., INA, K., OHMIYA, N., NIWA, Y. & GOTO, H. (2008) Helicobacter pylori infection upregulates interleukin-18 production from gastric epithelial cells. *European Journal of Gastroenterology & Hepatology,* 20, 1144-50.

SHIMIZU, T., HARUNA, H., OHTSUKA, Y., KANEKO, K., GUPTA, R. & YAMASHIRO, Y. (2004) Cytokines in the gastric mucosa of children with Helicobacter pylori infection. *Acta Paediatrica,* 93, 322-6.

SHINOHARA, M. L., KIM, J.-H., GARCIA, V. A. & CANTOR, H. (2008) Engagement of the type I interferon receptor on dendritic cells inhibits T helper 17 cell development: role of intracellular osteopontin. *Immunity,* 29, 68-78.

SHIOMI, S., TORIIE, A., IMAMURA, S., KONISHI, H., MITSUFUJI, S., IWAKURA, Y., YAMAOKA, Y., OTA, H., YAMAMOTO, T., IMANISHI, J. & KITA, M. (2008) IL-17 is involved in Helicobacter pylori-induced gastric inflammatory responses in a mouse model. *Helicobacter,* 13, 518-24.

SHIOTA, S., WATADA, M., MATSUNARI, O., IWATANI, S., SUZUKI, R. & YAMAOKA, Y. (2012) Helicobacter pylori iceA, clinical outcomes, and correlation with cagA: a meta-analysis. *PLoS ONE [Electronic Resource],* 7, e30354.

SHIOTANI, A., IISHI, H., UEDO, N., ISHIGURO, S., TATSUTA, M., NAKAE, Y., KUMAMOTO, M. & MERCHANT, J. L. (2005) Evidence that loss of sonic hedgehog is an indicator of Helicobater pylori-induced atrophic gastritis progressing to gastric cancer. *American Journal of Gastroenterology,* 100, 581-7.

SHORTMAN, K. & LIU, Y.-J. (2002) Mouse and human dendritic cell subtypes. *Nature Reviews. Immunology,* 2, 151-61.

SMYTHIES, L. E., WAITES, K. B., LINDSEY, J. R., HARRIS, P. R., GHIARA, P. & SMITH, P. D. (2000) *Helicobacter pylori*-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-γ, gene-deficient mice. *The Journal of Immunology,* 165, 1022-29.

SNIJDERS, A., HILKENS, C. M., VAN DER POUW KRAAN, T. C., ENGEL, M., AARDEN, L. A. & KAPSENBERG, M. L. (1996) Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. *Journal of Immunology,* 156, 1207-12.

SOARES, T. F., ROCHA, G. A., ROCHA, A. M. C., CORREA-OLIVEIRA, R., MARTINS-FILHO, O. A., CARVALHO, A. S. T., BITTENCOURT, P., OLIVEIRA, C. A., FARIA, A. M. C. & QUEIROZ, D. M. M. (2005) Phenotypic study of peripheral blood lymphocytes and humoral immune response in Helicobacter pylori infection according to age. *Scandinavian Journal of Immunology,* 62, 63-70.

SOMMER, F., FALLER, G., KONTUREK, P., KIRCHNER, T., HAHN, E. G., ZEUS, J., ROLLINGHOFF, M. & LOHOFF, M. (1998) Antrum- and corpus mucosa-infiltrating $CD4^+$ lymphocytes in *Helicobacter pylori* gastritis display a Th1 phenotype. *Infection and Immunity,* 66, 5543-46.

SPITS, H., ARTIS, D., COLONNA, M., DIEFENBACH, A., DI SANTO, J. P., EBERL, G., KOYASU, S., LOCKSLEY, R. M., MCKENZIE, A. N. J., MEBIUS, R. E., POWRIE, F. & VIVIER, E. (2013) Innate lymphoid cells - a proposal for uniform nomenclature. *Nat Rev Immunol,* 13, 145-149.

SPOLSKI, R. & LEONARD, W. J. IL-21 and T follicular helper cells. *International Immunology,* 22, 7-12.

STASI, R., SARPATWARI, A., SEGAL, J. B., OSBORN, J., EVANGELISTA, M. L., COOPER, N., PROVAN, D., NEWLAND, A., AMADORI, S. & BUSSEL, J. B. (2009) Effects of eradication of Helicobacter pylori infection in patients with immune thrombocytopenic purpura: a systematic review. *Blood,* 113, 1231-40.

STEEVENS, J., SCHOUTEN, L. J., GOLDBOHM, R. A. & VAN DEN BRANDT, P. A. (2010) Alcohol consumption, cigarette smoking and risk of subtypes of oesophageal and gastric cancer: a prospective cohort study. *Gut,* 59, 39-48.

STEINER, G. E., NEWMAN, M. E., PAIKL, D., STIX, U., MEMARAN-DAGDA, N., LEE, C. & MARBERGER, M. J. (2003) Expression and function of pro-inflammatory interleukin IL-17 and IL-17 receptor in normal, benign hyperplastic, and malignant prostate. *The Prostate,* 56, 171-182.

STOICOV, C., FAN, X., LIU, J. H., BOWEN, G., WHARY, M., KURT-JONES, E. & HOUGHTON, J. (2009) T-bet Knockout Prevents Helicobacter felis-Induced Gastric Cancer. *The Journal of Immunology,* 183, 642-649.

STRACHAN, D. P. (1989) Hay fever, hygiene, and household size. *BMJ,* 299, 1259-60.

STREECK, H., COHEN, K. W., JOLIN, J. S., BROCKMAN, M. A., MEIER, A., POWER, K. A., WARING, M. T., ALTER, G. & MARCUS, A. (2008) Rapid ex vivo isolation and long-term culture of human Th17 cells. *Journal of Immunological Methods.*

STUMHOFER, J. S., SILVER, J. S., LAURENCE, A., PORRETT, P. M., HARRIS, T. H., TURKA, L. A., ERNST, M., SARIS, C. J. M., O'SHEA, J. J. & HUNTER, C. A. (2007) Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nature Immunology,* 8, 1363-71.

SU, X., YE, J., HSUEH, E. C., ZHANG, Y., HOFT, D. F. & PENG, G. (2010) Tumor microenvironments direct the recruitment and expansion of human Th17 cells. *Journal of Immunology,* 184, 1630-41.

SUERBAUM, S., JOSENHANS, C. & LABIGNE, A. (1993) Cloning and genetic characterization of the Helicobacter pylori and Helicobacter mustelae flaB flagellin genes and construction of H. pylori flaA- and flaB-negative mutants by electroporation-mediated allelic exchange. *Journal of Bacteriology,* 175, 3278-3288.

SUERBAUM, S. & MICHETTI, P. (2002) Helicobacter pylori infection. *New England Journal of Medicine,* 347, 1175-86.

SUGANUMA, M., KURUSU, M., SUZUKI, K., NISHIZONO, A., MURAKAMI, K., FUJIOKA, T. & FUJIKI, H. (2005) New tumor necrosis factor-alpha-inducing protein released from Helicobacter pylori for gastric cancer progression. *Journal of Cancer Research & Clinical Oncology,* 131, 305-13.

SUGANUMA, M., YAMAGUCHI, K., ONO, Y., MATSUMOTO, H., HAYASHI, T., OGAWA, T., IMAI, K., KUZUHARA, T., NISHIZONO, A. & FUJIKI, H. (2008) TNF-alpha-inducing protein, a carcinogenic factor secreted from H. pylori, enters gastric cancer cells. *International Journal of Cancer,* 123, 117-22.

SUGIMOTO, K., OGAWA, A., MIZOGUCHI, E., SHIMOMURA, Y., ANDOH, A., BHAN, A. K., BLUMBERG, R. S., XAVIER, R. J. & MIZOGUCHI, A. (2008) IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *Journal of Clinical Investigation,* 118, 534-44.

SUGIMOTO, M., FURUTA, T. & YAMAOKA, Y. (2009) Influence of inflammatory cytokine polymorphisms on eradication rates of Helicobacter pylori. *Journal of Gastroenterology & Hepatology,* 24, 1725-32.

SUN, C.-M., HALL, J. A., BLANK, R. B., BOULADOUX, N., OUKKA, M., MORA, J. R. & BELKAID, Y. (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *Journal of Experimental Medicine,* 204, 1775-85.

SUN, H. Q., ZHANG, J. Y., ZHANG, H., ZOU, Z. S., WANG, F. S. & JIA, J. H. (2012) Increased Th17 cells contribute to disease progression in patients with HBV-associated liver cirrhosis. *Journal of Viral Hepatitis,* 19, 396-403.

SUNDRUD, M. S., KORALOV, S. B., FEUERER, M., CALADO, D. P., KOZHAYA, A. E., RHULE-SMITH, A., LEFEBVRE, R. E., UNUTMAZ, D., MAZITSCHEK, R., WALDNER, H., WHITMAN, M., KELLER, T. & RAO, A. (2009) Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response.[see comment]. *Science,* 324, 1334-8.

SUTTON, C. E., LALOR, S. J., SWEENEY, C. M., BRERETON, C. F., LAVELLE, E. C. & MILLS, K. H. G. (2009) Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity,* 31, 331-41.

SUZUKI, T., MATSUSHIMA, M., MASUI, A., WATANABE, K.-I., TAKAGI, A., OGAWA, Y., SHIRAI, T. & MINE, T. (2005) Effect of Helicobacter pylori eradication in patients with chronic idiopathic thrombocytopenic purpura-a randomized controlled trial. *American Journal of Gastroenterology,* 100, 1265-70.

TAJIMA, M., WAKITA, D., NOGUCHI, D., CHAMOTO, K., YUE, Z., FUGO, K., ISHIGAME, H., IWAKURA, Y., KITAMURA, H. & NISHIMURA, T. (2008) IL-6-dependent spontaneous proliferation is required for the induction of colitogenic IL-17-producing CD8+ T cells. *Journal of Experimental Medicine,* 205, 1019-27.

TAKAHASHI, N., VANLAERE, I., DE RYCKE, R., CAUWELS, A., JOOSTEN, L. A. B., LUBBERTS, E., VAN DEN BERG, W. B. & LIBERT, C. (2008) IL-17 produced by Paneth cells drives TNF-induced shock. *Journal of Experimental Medicine,* 205, 1755-61.

TAKAHASHI, T., YUJIRI, T., SHINOHARA, K., INOUE, Y., SATO, Y., FUJII, Y., OKUBO, M., ZAITSU, Y., ARIYOSHI, K., NAKAMURA, Y., NAWATA, R., OKA, Y., SHIRAI, M. & TANIZAWA, Y. (2004) Molecular mimicry by Helicobacter pylori CagA protein may be involved in the pathogenesis of H. pylori-associated chronic idiopathic thrombocytopenic purpura. *British Journal of Haematology,* 124, 91-6.

TAKASHIMA, M., FURUTA, T., HANAI, H., SUGIMURA, H. & KANEKO, E. (2001) Effects of Helicobacter pylori infection on gastric acid secretion and serum gastrin levels in Mongolian gerbils. *Gut,* 48, 765-73.

TAKATORI, H., KANNO, Y., WATFORD, W. T., TATO, C. M., WEISS, G., IVANOV, I. I., LITTMAN, D. R. & O'SHEA, J. J. (2009) Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *Journal of Experimental Medicine,* 206, 35-41.

TAN, H.-J. & GOH, K.-L. (2012) Extragastrointestinal manifestations of Helicobacter pylori infection: facts or myth? A critical review. *Journal of Digestive Diseases,* 13, 342-9.

TAY, C. Y., WINDSOR, H. M., THIRRIOT, F., LU, W., CONWAY, C., PERKINS, T. T. & MARSHALL, B. J. (2012) Helicobacter pylori eradication in Western Australia using novel quadruple therapy combinations. *Alimentary Pharmacology & Therapeutics,* 36, 1076-1083.

TAYLOR, S., WAKEM, M., DIJKMAN, G., ALSARRAJ, M. & NGUYEN, M. (2010) A practical approach to RT-qPCR: Publishing data that conform to the MIQE guidelines. *Methods,* 50, S1-S5.

THOMAS, J. E., BUNN, J. E., PIPER, J., KLEANTHOUS, H., MONATH, T. P., HARDING, M., COWARD, W. A. & WEAVER, L. T. (1998) Specific anti-H. pylori urease IgA in human Breastmilk and delayed H. pylori colonisation in Gambian infants. *Journal of Pediatric Gastroenterology and Nutrition,* 26, 567.

THORNTON, A. M., KORTY, P. E., TRAN, D. Q., WOHLFERT, E. A., MURRAY, P. E., BELKAID, Y. & SHEVACH, E. M. (2010) Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *Journal of Immunology,* 184, 3433-41.

TOMITA, T., JACKSON, A. M., HIDA, N., HAYAT, M., DIXON, M. F., SHIMOYAMA, T., AXON, A. T., ROBINSON, P. A. & CRABTREE, J. E. (2001) Expression of Interleukin-18, a Th1 cytokine, in human gastric mucosa is increased in Helicobacter pylori infection. *Journal of Infectious Diseases,* 183, 620-7.

TORCHINSKY, M. B., GARAUDE, J., MARTIN, A. P. & BLANDER, J. M. (2009) Innate immune recognition of infected apoptotic cells directs TH17 cell differentiation. *Nature,* 458, 78-82.

TREJDOSIEWICZ, L. K., CALABRESE, A., SMART, C. J., OAKES, D. J., HOWDLE, P. D., CRABTREE, E., LOSOWSKY, M. S., LANCASTER, F. & BOYLSTON, A. W. (1991) Gamma delta T cell receptor-positive cells of the human gastrointestinal mucosa: occurrence and V region gene expression in Heliobacter pylori-associated gastritis, coeliac disease and inflammatory bowel disease. *Clinical & Experimental Immunology,* 84, 440-4.

TURE-OZDEMIR, F., GAZOULI, M., TZIVRAS, M., PANAGOS, C., BOVARETOS, N., PETRAKI, K., GIANNAKOPOULOS, A., KORKOLOPOULOU, P. & MANTZARIS, G. J. (2008) Association of polymorphisms of NOD2, TLR4 and CD14 genes with susceptibility to gastric mucosa-associated lymphoid tissue lymphoma. *Anticancer Research,* 28, 3697-700.

UEHARA, A., OKUMURA, T., SEKIYA, C., OKAMURA, K., TAKASUGI, Y. & NAMIKI, M. (1989) Interleukin-1 inhibits the secretion of gastric acid in rats: possible involvement of prostaglandin. *Biochemical & Biophysical Research Communications,* 162, 1578-84.

UMEMURA, M., YAHAGI, A., HAMADA, S., BEGUM, M. D., WATANABE, H., KAWAKAMI, K., SUDA, T., SUDO, K., NAKAE, S., IWAKURA, Y. & MATSUZAKI, G. (2007) IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. *Journal of Immunology,* 178, 3786-96.

VAN BEELEN, A. J., ZELINKOVA, Z., TANAANMAN-KUETER, E. W., MULLER, F. J., HOMMES, D. W., ZAAT, S. A. J., KAPSENBERG, M. L. & DE JONG, E. C. (2007) Stimulation of the Intracellular Bacterial Sensor NOD2 Programs Dendritic Cells to Promote Interleukin-17 Production in Human Memory T Cells. *Immunity,* 27, 660-669.

VELDHOEN, M., HIROTA, K., CHRISTENSEN, J., O'GARRA, A. & STOCKINGER, B. (2009) Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. *Journal of Experimental Medicine,* 206, 43-9.

VELDHOEN, M., HIROTA, K., WESTERNDORF, A. M., BUER, J., DUMOUTIER, L., RENAULD, J.-C. & STOCKINGER, B. (2008a) The aryl hydrocarbon receptor links Th17-cell-mediated autoimmunity to environmental toxins. *Nature,* 453, 106-110.

VELDHOEN, M., HOCKING, R. J., ATKINS, C. J., LOCKSLEY, R. M. & STOCKINGER, B. (2006) TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity,* 24, 179-89.

VELDHOEN, M., UYTTENHOVE, C., VAN SNICK, J., HELMBY, H., WESTENDORF, A., BUER, J., MARTIN, B., WILHELM, C. & STOCKINGER, B. (2008b) Transforming growth factor-β 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol,* 9, 1341-1346.

VELIN, D., BACHMANN, D., BOUZOURENE, H. & MICHETTI, P. (2005) Mast Cells Are Critical Mediators of Vaccine-Induced Helicobacter Clearance in the Mouse Model. *Gastroenterology,* 129, 142-155.

VELIN, D., FAVRE, L., BERNASCONI, E., BACHMANN, D., PYTHOUD, C., SAIJI, E., BOUZOURENE, H. & MICHETTI, P. (2009) Interleukin-17 Is a Critical Mediator of Vaccine-Induced Reduction of Helicobacter Infection in the Mouse Model. *Gastroenterology,* 136, 2237-2246.e1.

VIALA, J., CHAPUT, C., BONECA, I. G., CARDONA, A., GIRARDIN, S. E., MORAN, A. P., ATHMAN, R., MEMET, S., HUERRE, M. R., COYLE, A. J., DISTEFANO, P. S., SANSONETTI, P. J., LABIGNE, A., BERTIN, J., PHILPOTT, D. J. & FERRERO, R. L. (2004) Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nature Immunology,* 5, 1166-74.

VIANA, P. O., ONO, E., MIYAMOTO, M., SALOMAO, R., COSTA-CARVALHO, B. T., WECKX, L. Y. & DE MORAES-PINTO, M. I. Humoral and cellular immune responses to measles and tetanus: the importance of elapsed time since last exposure and the nature of the antigen. *Journal of Clinical Immunology,* 30, 574-82.

VOKAER, B., VAN ROMPAEY, N., LEMAITRE, P. H., LHOMME, F., KUBJAK, C., BENGHIAT, F. S., IWAKURA, Y., PETEIN, M., FIELD, K. A., GOLDMAN, M., LE MOINE, A. & CHARBONNIER, L.-M. (2010) Critical role of regulatory T cells in Th17-mediated minor antigen-disparate rejection. *Journal of Immunology,* 185, 3417-25.

VOLPE, E., SERVANT, N., ZOLLINGER, R. L., BOGIATZI, S. I., HUPE´, P., BARILLOT, E. & SOUMELIS, V. (2008) A critical function for transforming growth factor-b, interleukir 23 and proinflammatory cytokines in driving and modulating human T_H-17 responses. *Nature Immunology,* 9, 650-7.

VONARBOURG, C., MORTHA, A., BUI, V. L., HERNANDEZ, P. P., KISS, E. A., HOYLER, T., FLACH, M., BENGSCH, B., THIMME, R., HÃSCHER, C., HÃNIG, M., PANNICKE, U., SCHWARZ, K WARE, C. F., FINKE, D. & DIEFENBACH, A. (2010) Regulated Expression of Nuclear Receptor RORgt Confers Distinct Functional Fates to NK Cell Receptor-Expressing RORgt+ Innate Lymphocytes. *Immunity,* 33, 736-751.

VOO, K. S., WANG, Y.-H., SANTORI, F. R., BOGGIANO, C., WANG, Y.-H., ARIMA, K., BOVER, L., HANABUCHI, S., KHALILI, J., MARINOVA, E., ZHENG, B., LITTMAN, D. R. & LIU, Y.-J. (2009) Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proceedings of the National Academy of Sciences of the United States of America,* 106, 4793-8.

WAGHRAY, M., ZAVROS, Y., SAQUI-SALCES, M., EL-ZAATARI, M., ALAMELUMANGAPURAM, C. B., TODISCO, A., EATON, K. A. & MERCHANT, J. L. Interleukin-1beta promotes gastric atrophy through suppression of Sonic Hedgehog. *Gastroenterology,* 138, 562-72.

WALKER, J. A., BARLOW, J. L. & MCKENZIE, A. N. J. (2013) Innate lymphoid cells - how did we miss them? *Nat Rev Immunol,* 13, 75-87.

WALKER, M. R., KASPROWICZ, D. J., GERSUK, V. H., BENARD, A., VAN LANDEGHEN, M., BUCKNER, J. H. & ZIEGLER, S. F. (2003) Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *Journal of Clinical Investigation,* 112, 1437-43.

WANG, H. H., DAI, Y. Q., QIU, W., LU, Z. Q., PENG, F. H., WANG, Y. G., BAO, J., LI, Y. & HU, X. Q. (2011) Interleukin-17-secreting T cells in neuromyelitis optica and multiple sclerosis during relapse. *Journal of Clinical Neuroscience,* 18, 1313-7.

WANG, H. Y. & WANG, R.-F. (2005) Antigen-specific CD4+ regulatory T cells in cancer: implications for immunotherapy. *Microbes and Infection,* 7, 1056-62.

WANG, J., IOAN-FACSINAY, A., VAN DER VOORT, E. I. H., HUIZINGA, T. W. J. & TOES, R. E. M. (2007a) Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *European Journal of Immunology,* 37, 129-38.

WANG, L., YI, T., KORTYLEWSKI, M., PARDOLL, D. M., ZENG, D. & YU, H. (2009) IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *Journal of Experimental Medicine,* 206, 1457-64.

WANG, S.-K., ZHU, H.-F., HE, B.-S., ZHANG, Z.-Y., CHEN, Z.-T., WANG, Z.-Z. & WU, G.-L. (2007b) CagA+ H pylori infection is associated with polarization of T helper cell immune responses in gastric carcinogenesis. *World Journal of Gastroenterology,* 13, 2923-31.

WANG, Y.-H., GORVEL, J.-P., CHU, Y.-T., WU, J.-J. & LEI, H.-Y. (2010) Helicobacter pylori impairs murine dendritic cell responses to infection. *PLoS ONE [Electronic Resource],* 5, e10844.

WEAVER, C. T., HAWRYLOWICZ, C. M. & UNANUE, E. R. (1988) T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. *Proceedings of the National Academy of Sciences,* 85, 8181-8185.

WEI, G., WEI, L., ZHU, J., ZANG, C., HU-LI, J., YAO, Z., CUI, K., KANNO, Y., ROH, T.-Y., WATFORD, W. T., SCHONES, D. E., PENG, W., SUN, H.-W., PAUL, W. E., O'SHEA, J. J. & ZHAO, K. (2009) Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity,* 30, 155-67.

WERNER, J. L., GESSNER, M. A., LILLY, L. M., NELSON, M. P., METZ, A. E., HORN, D., DUNAWAY, C. W., DESHANE, J., CHAPLIN, D. D., WEAVER, C. T., BROWN, G. D. & STEELE, C. (2011) Neutrophils Produce Interleukin 17A (IL-17A) in a Dectin-1- and IL-23-Dependent Manner during Invasive Fungal Infection. *Infection and Immunity,* 79, 3966-3977.

WEST, J., LOGAN, R., F. A. CHRIS, J. SMITH, HUBBARD, R., B. & CARD, T., R. (2004) Malignancy and mortality in people with coeliac disease: population based cohort study. *BMJ,* 329, 716-719.

WIEHLER, S. & PROUD, D. (2007) Interleukin-17A modulates human airway epithelial responses to human rhinovirus infection. *American Journal of Physiology - Lung Cellular & Molecular Physiology,* 293, L505-15.

WILSON, N. J., BONIFACE, K., CHAN, J. R., MCKENZIE, B. S., BLUMENSCHEIN, W. M., MATTSON, J. D., BASHAM, B., SMITH, K., CHEN, T., MOREL, F., LECRON, J.-C., KASTELEIN, R. A., CUA, D. J., MCCLANAHAN, T. K., BOWMAN, E. P. & DE WAAL MALEFYT, R. (2007) Development, cytokine profile and function of human interleukin 17–producing helper T cells. *Nature Immunology,* 8, 950-57.

WOLK, K., WITTE, E., WALLACE, E., DOCKE, W.-D., KUNZ, S., ASADULLAH, K., VOLK, H.-D., STERRY, W. & SABAT, R. (2006) IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. *European Journal of Immunology,* 36, 1309-23.

WOLK, K., WITTE, E., WITTE, K., WARSZAWSKA, K. & SABAT, R. Biology of interleukin-22. *Seminars In Immunopathology,* 32, 17-31.

WOLLE, K. & MALFERTHEINER, P. (2007) Treatment of Helicobacter pylori. *Best Practice & Research in Clinical Gastroenterology,* 21, 315-24.

WONG, B. C.-Y., LAM, S. K., WONG, W. M., CHEN, J. S., ZHENG, T. T., FENG, R. E., LAI, K. C., HU, W. H. C., YUEN, S. T., LEUNG, S. Y., FONG, D. Y. T., HO, J., CHING, C. K., CHEN, J. S. & CHINA GASTRIC CANCER STUDY, G. (2004) Helicobacter pylori eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. *JAMA,* 291, 187-94.

WONG, B. L. W., ZHU, S.-L., HUANG, X. R., MA, J., XIA, H. H. X., BUCALA, R., WONG, B. C. Y. & LAN, H. Y. (2009) Essential role for macrophage migration inhibitory factor in gastritis induced by Helicobacter pylori. *American Journal of Pathology,* 174, 1319-28.

WORTHLEY, D. L., RUSZKIEWICZ, A., DAVIES, R., MOORE, S., NIVISON-SMITH, I., BIK TO, L., BROWETT, P., WESTERN, R., DURRANT, S., SO, J., YOUNG, G. P., MULLIGHAN, C. G., BARDY, P. G. & MICHAEL, M. Z. (2009) Human Gastrointestinal Neoplasia-Associated Myofibroblasts Can Develop from Bone Marrow-Derived Cells Following Allogeneic Stem Cell Transplantation. *STEM CELLS,* 27, 1463-1468.

WOTHERSPOON, A. C., DOGLIONI, C., DISS, T. C., PAN, L., MOSCHINI, A., DE BONI, M. & ISAACSON, P. G. (1993) Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of Helicobacter pylori.[see comment]. *Lancet,* 342, 575-7.

WU, Y.-Y., TSAI, H.-F., LIN, W.-C., HSU, P.-I., SHUN, C.-T., WU, M.-S. & HSU, P.-N. (2007) Upregulation of CCL20 and recruitment of CCR6+ gastric infiltrating lymphocytes in Helicobacter pylori gastritis. *Infection & Immunity,* 75, 4357-63.

XIA, H. H.-X., LAM, S.-K., HUANG, X.-R., WONG, W.-M., LEUNG, S.-Y., YUEN, S.-T., LAN, H.-Y. & WONG, B. C.-Y. (2004) Helicobacter pylori infection is associated with increased expression of macrophage migratory inhibitory factor--by epithelial cells, T cells, and macrophages--in gastric mucosa. *Journal of Infectious Diseases,* 190, 293-302.

XIAO, B., LIU, Z., LI, B.-S., TANG, B., LI, W., GUO, G., SHI, Y., WANG, F., WU, Y., TONG, W.-D., GUO, H., MAO, X.-H. & ZOU, Q.-M. (2009) Induction of microRNA-155 during Helicobacter pylori Infection and Its Negative Regulatory Role in the Inflammatory Response. *Journal of Infectious Diseases,* 200, 916-925.

XU, J., YANG, Y., QIU, G., LAL, G., WU, Z., LEVY, D. E., OCHANDO, J. C., BROMBERG, J. S. & DING, Y. (2009) c-Maf regulates IL-10 expression during Th17 polarization. *Journal of Immunology,* 182, 6226-36.

XU, L., KITANI, A., FUSS, I. & STROBER, W. (2007) Cutting Edge: Regulatory T cells induce $CD4^{+}CD25^{-}Foxp3^{-}$ T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-β. *The Journal of Immunology,* 178, 6725-9.

YAMADA, H., NAKASHIMA, Y., OKAZAKI, K., MAWATARI, T., FUKUSHI, J.-I., OYAMADA, A., FUJIMURA, K., IWAMOTO, Y. & YOSHIKAI, Y. (2011) Preferential accumulation of activated Th1 cells not only in rheumatoid arthritis but also in osteoarthritis joints. *Journal of Rheumatology,* 38, 1569-75.

YAMAOKA, Y., KITA, M., KODAMA, T., SAWAI, N., KASHIMA, K. & IMANISHI, J. (1995) Expression of cytokine mRNA in gastric mucosa with Helicobacter pylori infection. *Scandinavian Journal of Gastroenterology,* 30, 1153-9.

YAMAOKA, Y., KITA, M., KODAMA, T., SAWAI, N., KASHIMA, K. & IMANISHI, J. (1997) Induction of various cytokines and development of severe mucosal inflammation by cagA gene positive Helicobacter pylori strains. *Gut,* 41, 442-51.

YAMAOKA, Y., KITA, M., KODAMA, T., SAWAI, N., TANAHASHI, T., KASHIMA, K. & IMANISHI, J. (1998) Chemokines in the gastric mucosa in Helicobacter pylori infection. *Gut,* 42, 609-17.

YAMAOKA, Y., KODAMA, T., KITA, M., IMANISHI, J., KASHIMA, K. & GRAHAM, D. Y. (1999) Relation between clinical presentation,Helicobacter pylori density, interleukin 1$\hat{1}^2$ and 8 production, and cagA status. *Gut,* 45, 804-811.

YAMAOKA, Y., KODAMA, T., KITA, M., IMANISHI, J., KASHIMA, K. & GRAHAM, D. Y. (2001) Relation between cytokines and Helicobacter pylori in gastric cancer. *Helicobacter,* 6, 116-24.

YAMAOKA, Y., KWON, D. H. & GRAHAM, D. Y. (2000) A Mr 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. *Proceedings of the National Academy of Sciences,* 97, 7533-7538.

YAMAOKA, Y., YAMAUCHI, K., OTA, H., SUGIYAMA, A., ISHIZONE, S., GRAHAM, D. Y., MARUTA, F., MURAKAMI, M. & KATSUYAMA, T. (2005) Natural history of gastric mucosal cytokine expression in Helicobacter pylori gastritis in Mongolian gerbils. *Infection & Immunity,* 73, 2205-12.

YAMAUCHI, K., CHOI, I.-J., LU, H., OGIWARA, H., GRAHAM, D. Y. & YAMAOKA, Y. (2008) Regulation of IL-18 in Helicobacter pylori infection. *Journal of Immunology,* 180, 1207-16.

YANG, D., CHEN, Q., HOOVER, D. M., STALEY, P., TUCKER, K. D., LUBKOWSKI, J. & OPPENHEIM, J. J. (2003) Many chemokines including CCL20/MIP-3a display antimicrobial activity. *Journal of Leukocyte Biology,* 74, 448-455.

YANG, D., CHERTOV, O., BYKOVSKAIA, S. N., CHEN, Q., BUFFO, M. J., SHOGAN, J., ANDERSON, M., SCHRÃDER, J. M., WANG, J. M., HOWARD, O. M. Z. & OPPENHEIM, J. J. (1999) b-Defensins: Linking Innate and Adaptive Immunity Through Dendritic and T Cell CCR6. *Science,* 286, 525-528.

YANG, L., ANDERSON, D. E., BAECHER-ALLAN, C., HASTINGS, W. D., BETTELLI, E., OUKKA, M., KUCHROO, V. K. & HAFLER, D. A. (2008a) IL-21 and TGF-b are required for differentiation of human TH17 cells. *Nature,* 454, 350-352.

YANG, X. O., NURIEVA, R., MARTINEZ, G. J., KANG, H. S., CHUNG, Y., PAPPU, B. P., SHAH, B., CHANG, S. H., SCHLUNS, K. S., WATOWICH, S. S., FENG, X.-H., JETTEN, A. M. & DONG, C. (2008b) Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity,* 29, 44-56.

YANG, X. O., PAPPU, B. P., NURIEVA, R., AKIMZHANOV, A., KANG, H. S., CHUNG, Y., MA, L., SHAH, B., PANOPOULOS, A. D., SCHLUNS, K. S., WATOWICH, S. S., TIAN, Q., JETTEN, A. M. & DONG, C. (2008c) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity,* 28, 29-39.

YE, P., RODRIGUEZ, F. H., KANALY, S., STOCKING, K. L., SCHURR, J., SCHWARZENBERGER, P., OLIVER, P., HUANG, W., ZHANG, P., ZHANG, J., SHELLITO, J. E., BAGBY, G. J., NELSON, S., CHARRIER, K., PESCHON, J. J. & KOLLS, J. K. (2001) Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *Journal of Experimental Medicine,* 194, 519-27.

YE, Z.-J., ZHOU, Q., GU, Y.-Y., QIN, S.-M., MA, W.-L., XIN, J.-B., TAO, X.-N. & SHI, H.-Z. (2010) Generation and differentiation of IL-17-producing CD4+ T cells in malignant pleural effusion. *Journal of Immunology,* 185, 6348-54.

YEN, H.-R., HARRIS, T. J., WADA, S., GROSSO, J. F., GETNET, D., GOLDBERG, M. V., LIANG, K.-L., BRUNO, T. C., PYLE, K. J., CHAN, S.-L., ANDERS, R. A., TRIMBLE, C. L., ADLER, A. J., LIN, T.-Y., PARDOLL, D. M., HUANG, C.-T. & DRAKE, C. G. (2009) Tc17 CD8 T cells: functional plasticity and subset diversity. *Journal of Immunology,* 183, 7161-8.

YOSHIDA, A., ISOMOTO, H., HISATSUNE, J., NAKAYAMA, M., NAKASHIMA, Y., MATSUSHIMA, K., MIZUTA, Y., HAYASHI, T., YAMAOKA, Y., AZUMA, T., MOSS, J., HIRAYAMA, T. & KOHNO, S. (2009) Enhanced expression of CCL20 in human Helicobacter pylori-associated gastritis. *Clinical Immunology,* 130, 290-7.

YOSHIDA, M., KOBAYASHI, K., KUO, T. T., BRY, L., GLICKMAN, J. N., CLAYPOOL, S. M., KASER, A., NAGAISHI, T., HIGGINS, D. E., MIZOGUCHI, E., WAKATSUKI, Y., ROOPENIAN, D. C., MIZOGUCHI, A., LENCER, W. I. & BLUMBERG, R. S. (2006) Neonatal Fc receptor for IgG regulates mucosal immune responses to luminal bacteria. *Journal of Clinical Investigation,* 116, 2142-2151.

YUAN, W., LI, Y., YANG, K., MA, B., GUAN, Q., WANG, D. & YANG, L. (2010) Iron deficiency anemia in Helicobacter pylori infection: meta-analysis of randomized controlled trials. *Scandinavian Journal of Gastroenterology,* 45, 665-76.

YUE, F. Y., MERCHANT, A., KOVACS, C. M., LOUTFY, M., PERSAD, D. & OSTROWSKI, M. A. (2008) Virus-specific interleukin-17-producing CD4+ T cells are detectable in early human immunodeficiency virus type 1 infection. *Journal of Virology,* 82, 6767-71.

ZASLOFF, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature,* 415, 389-395.

ZAVROS, Y. & MERCHANT, J. L. (2005) Modulating the cytokine response to treat Helicobacter gastritis. *Biochemical Pharmacology,* 69, 365-371.

ZENEWICZ, L. A., YANCOPOULOS, G. D., VALENZUELA, D. M., MURPHY, A. J., KAROW, M. & FLAVELL, R. A. (2007) Interleukin-22 but Not Interleukin-17 Provides Protection to Hepatocytes during Acute Liver Inflammation. *Immunity,* 27, 647-59.

ZENEWICZ, L. A., YANCOPOULOS, G. D., VALENZUELA, D. M., MURPHY, A. J., STEVENS, S. & FLAVELL, R. A. (2008) Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity,* 29, 947-57.

ZHANG, B., RONG, G., WEI, H., ZHANG, M., BI, J., MAA, L., XUE, X., WEI, G., LIU, X. & FANG, G. (2008a) The prevalence of Th17 cells in patients with gastric cancer. *Biochemical and Biophysical Research Communications,* 374, 533-37.

ZHANG, H., FANG, D.-C., LAN, C.-H. & LUO, Y.-H. (2007) Helicobacter pylori infection induces apoptosis in gastric cancer cells through the mitochondrial pathway. *Journal of Gastroenterology & Hepatology,* 22, 1051-6.

ZHANG, J.-P., YAN, J., XU, J., PANG, X.-H., CHEN, M.-S., LI, L., WU, C., LI, S.-P. & ZHENG, L. (2009) Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. *Journal of Hepatology,* 50, 980-9.

ZHANG, J.-Y., LIU, T., GUO, H., LIU, X.-F., ZHUANG, Y., YU, S., CHEN, L., WU, C., ZHAO, Z., TANG, B., LUO, P., MAO, X.-H., GUO, G., SHI, Y. & ZOU, Q.-M. (2011) Induction of a Th17 cell response by Helicobacter pylori Urease subunit B. *Immunobiology,* 216, 803-10.

ZHANG, J.-Y., ZHANG, Z., LIN, F., ZOU, Z.-S., XU, R.-N., JIN, L., FU, J.-L., SHI, F., SHI, M., WANG, H.-F. & WANG, F.-S. Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. *Hepatology,* 51, 81-91.

ZHANG, L., YANG, X.-Q., CHENG, J., HUI, R.-S. & GAO, T.-W. (2010a) Increased Th17 cells are accompanied by FoxP3(+) Treg cell accumulation and correlated with psoriasis disease severity. *Clinical Immunology,* 135, 108-17.

ZHANG, M., MAOCHANG, L., LUTHER, J. & KAO, J. Y. (2010b) *Helicobacter pylori* directs tolerogenic programming of dendritic cells. *Gut Microbes,* 1, 325-329.

ZHANG, X., JIN, J., PENG, X., RAMGOLAM, V. S. & MARKOVIC-PLESE, S. (2008b) Simvastatin inhibits IL-17 secretion by targeting multiple IL-17-regulatory cytokines and by inhibiting the expression of IL-17 transcription factor RORC in CD4+ lymphocytes. *Journal of Immunology,* 180, 6988-96.

ZHANG, Y.-L., LI, J., MO, H.-Y., QIU, F., ZHENG, L.-M., QIAN, C.-N. & ZENG, Y.-X. (2010c) Different subsets of tumor infiltrating lymphocytes correlate with NPC progression in different ways. *Molecular Cancer,* 9, 4.

ZHANG, Z. W. & FARTHING, M. J. G. (2005) The roles of vitamin C in Helicobacter pylori associated gastric carcinogenesis. *Chinese Journal of Digestive Diseases,* 6, 53-8.

ZHOU, G. & LEVITSKY, H. I. (2007) Natural Regulatory T Cells and De Novo-Induced Regulatory T Cells Contribute Independently to Tumor-Specific Tolerance. *The Journal of Immunology,* 178, 2155-2162.

ZHOU, L., IVANOV, I. I., SPOLSKI, R., MIN, R., SHENDEROV, K., EGAWA, T., LEVY, D. E., LEONARD, W. J. & LITTMAN, D. R. (2007a) IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol,* 8, 967-974.

ZHOU, L. & LITTMAN, D. R. (2009) Transcriptional regulatory networks in Th17 cell differentiation. *Current Opinion in Immunology,* 21, 146-52.

ZHOU, L., LOPES, J. E., CHONG, M. M. W., IVANOV, I. I., MIN, R., VICTORA, G. D., SHEN, Y., DU, J., RUBTSOV, Y. P., RUDENSKY, A. Y., ZIEGLER, S. F. & LITTMAN, D. R. (2008a) TGF-β-induced Foxp3 inhibits Th17 cell differentiation by antagonizing RORγt function. *Nature,* 1-6.

ZHOU, M., YANG, B., MA, R. & WU, C. (2008b) Memory Th-17 cells specific for C. albicans are persistent in human peripheral blood. *Immunology Letters,* 118, 72-81.

ZHOU, Y., TOH, M.-L., ZRIOUAL, S. & MIOSSEC, P. (2007b) IL-17A versus IL-17F induced intracellular signal transduction pathways and modulation by IL-17RA and IL-17RC RNA interference in AGS gastric adenocarcinoma cells. *Cytokine,* 38, 157-164.

ZHU, J. & PAUL, W. E. (2010) Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. *Immunological Reviews,* 238, 247-262.

ZHU, X., MULCAHY, L., MOHAMMED, R., LEE, A., FRANKS, H., KILPATRICK, L., YILMAZER, A., PAISH, E., ELLIS, I., PATEL, P. & JACKSON, A. (2008) IL-17 expression by breast-cancer-associated macrophages: IL-17 promotes invasiveness of breast cancer cell lines. *Breast Cancer Research,* 10, 1-11.

ZHUANG, Y., SHI, Y., LIU, X.-F., ZHANG, J.-Y., LIU, T., FAN, X., LUO, J., WU, C., YU, S., CHEN, L., LUO, P., GUO, G., LIU, Z., TANG, B., MAO, X.-H., GUO, Y. & ZOU, Q.-M. Helicobacter pylori-infected macrophages induce Th17 cell differentiation. *Immunobiology,* 216, 200-7.

ZIEGLER-HEITBROCK, L., ANCUTA, P., CROWE, S., DALOD, M., GRAU, V., HART, D. N., LEENEN, P. J. M., LIU, Y.-J., MACPHERSON, G., RANDOLPH, G. J., SCHERBERICH, J., SCHMITZ, J., SHORTMAN, K., SOZZANI, S., STROBL, H., ZEMBALA, M., AUSTYN, J. M. & LUTZ, M. B. (2010) Nomenclature of monocytes and dendritic cells in blood. *Blood,* 116, e74-80.

ZIELINSKI, C. E., MELE, F., ASCHENBRENNER, D., JARROSSAY, D., RONCHI, F., GATTORNO, M., MONTICELLI, S., LANZAVECCHIA, A. & SALLUSTO, F. (2012) Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature,* 484, 514-8.

ZULLO, A., HASSAN, C., CRISTOFARI, F., ANDRIANI, A., DE FRANCESCO, V., IERARDI, E., TOMAO, S., STOLTE, M., MORINI, S. & VAIRA, D. (2010) Effects of Helicobacter pylori eradication on early stage gastric mucosa-associated lymphoid tissue lymphoma. *Clinical Gastroenterology & Hepatology,* 8, 105-10.